

THERAPEUTIC TREATMENT AND
PREVENTION OF INFECTIONS WITH A BIOACTIVE MATERIALS
ENCAPSULATED WITHIN A BIODEGRADABLE-BIOCOMPATIBLE
POLYMERIC MATRIX

I. GOVERNMENT INTEREST

The invention described herein may be manufactured, used and licensed by or for the Government for Governmental purposes without the payment to use of any royalties thereon.

II. CROSS REFERENCE

This application is a continuation-in-part of U.S. Patent Application Serial No. 08/590,973 filed January 24, 1996 ^{dr 95} which in turn is a continuation-in-part of U.S. Patent Application Serial No. 08/446,149 filed May 22, 1995 which in turn is a continuation of U.S. Patent Application Serial No. 590,308 dated March 16, 1984.

Additionally, this application is a continuation-in-part of U.S. Patent Application Serial No. 08/446,148 filed May 22, 1995, which in turn is a continuation-in-part of U.S. Patent Application Serial No. 08/867,301 filed April 10, 1992 now U.S. Patent No. 5,417,986 issued May 23, 1995, which in turn is a continuation-in-part of U.S. Patent Application Serial No. 590,308 filed March 16, 1984.

III. FIELD OF THE INVENTION

1 This invention relates to compositions comprising active core
2 material(s) such as biologically active agent(s), drug(s) or substance(s)
3 encapsulated within an end-capped or a blend of uncapped and end-capped
4 biodegradable-biocompatible poly(lactide/glycolide) polymeric matrix useful
5 for the effective prevention or treatment of bacterial, viral, fungal, or parasitic
6 infections, and combinations thereof. In the areas of general and orthopedic
7 surgery, and the treatment of patients with infectious or chronic disease
8 conditions, this invention will be especially useful to physicians, dentists and
9 veterinarians.

0 IV. BACKGROUND OF THE INVENTION

1 Wounds characterized by the presence of infection, devitalized tissue,
2 and foreign-body contaminants have high infection rates and are difficult to
3 treat.

4 To prevent infection, in bone and soft tissue systemic antibiotics must
15 be administered within 4 hours after wounding when circulation is optimal.
16 This has been discussed by J.F. Burke in the article entitled "The Effective
17 Period of Preventive Antibiotic Action in Experimental Incisions and Dermal
18 Lesions", Surgery, Vol. 50, Page 161 (1961). If treatment of bacterial
19 infections is delayed, a milieu for bacterial growth develops which results in
20 complications associated with established infections. (G. Rodeheaver et al.,
21 "Proteolytic Enzymes as Adjuncts to Antibiotic Prophylaxis of Surgical
22 Wounds", American Journal of Surgery, Vol. 127, Page 564 (1974)). Once
23 infections are established it becomes difficult to systemically administer certain
24 antibiotics for extended periods at levels that are safe and effective at the

1 wound site. Unless administered locally, drugs are distributed throughout the
2 body, and the amount of drug hitting its target is only a small part of the total
3 dose. This ineffective use of the drug is compounded in the trauma patient by
4 hypovolemic shock, which results in a decreased vascular flow to tissues. (L.
5 E. Gelin et al., "Trauma Workshop Report: Shock rheology and Oxygen
6 Transport", Journal Trauma, Vol. 10, Page 1078 (1970)).

7 Additionally, infections caused by multiple-antibiotic resistant bacterial
8 are on the up-swing and we are on the verge of a potential world-wide medical
9 disaster. According to the Centers for Disease Control, 13,300 patients died
10 in U.S. hospitals in 1992 from infections caused by antibiotic-resistant
11 bacteria. Methicillin-resistant *S. aureus* (MRSA) is rapidly emerging as the
12 "pathogen of the 90's":

13 a. Some major teaching hospitals in U.S. report that up to 40%
14 of strains of *S. aureus* isolated from patients are resistant to methicillin. Many
15 of these MRSA strains are susceptible only to a single antibiotic (vancomycin).

16 b. Should MRSA also develop resistance to vancomycin, the
17 mortality rate among patients who develop MRSA infections could approach
18 80%, thereby increasing the threat of this infectious killer.

19 Moreover, Vancomycin resistance is on the up-swing:

20 a. 20% of Enterococci are now resistant to vancomycin

21 b. In 1989, only one hospital in New York City reported
22 vancomycin-resistant Enterococci. By 1991, the number of hospitals reporting
23 vancomycin resistance rose to 38.

1 c. transfer of vancomycin-resistant gene (via plasmid) has been
2 shown experimentally between Enterococcus and S. aureus.

3 Many major pharmaceutical companies around the world have either
4 completely eliminated or significantly reduced their research and development
5 programs in the area of antibiotic research. According to a 1994 report by the
6 Rockefeller University Workshop in Multiple Antibiotic Resistant Bacteria, we
7 are on the verge of a "medical disaster that would return physicians back to the
8 pre-penicillin days when even small infections could turn lethal due to the lack
9 of effective drugs."

10 Despite recent advances in antimicrobial therapy and improved surgical
11 techniques, osteomyelitis (hard tissue or bone infection) is still a source of
12 morbidity often necessitating lengthy hospitalization. The failure of patients
13 with chronic osteomyelitis to respond uniformly to conventional treatment has
14 prompted the search for more effective treatment modalities. Local antibiotic
15 therapy with gentamicin-impregnated poly(methylmethacrylate) (PMMA) bead
16 chains (SEPTOPAL TM, E. Merck, West Germany) has been utilized in
17 Germany for the treatment of osteomyelitis for the past decade and has been
18 reported to be efficacious in several clinical studies. The beads are implanted
19 into the bone at the time of surgical intervention where they provide
20 significantly higher concentrations of gentamicin than could otherwise be
21 achieved via systemic administration. Serum gentamicin levels, on the other
22 hand, remain extremely low thereby significantly reducing the potential for
23 nephro- and ototoxicity that occurs in some patients receiving gentamicin
24 systemically.

1 Since SEPTOPAL TM is not currently approved by the Food and Drug
2 Administration for use in the United States, some orthopedic surgeons in this
3 country are fabricating their own "physician-made beads" for the treatment of
4 chronic osteomyelitis. A major disadvantage of the beads, however, is that
5 because the PMMA is not biodegradable it represents a foreign body and
6 should be removed at about 2-weeks postimplantation thereby necessitating in
7 some cases an additional surgical procedure. A biodegradable-biocompatible,
8 antibiotic carrier, on the other hand, would eliminate the need for this
9 additional surgical procedure and may potentially reduce both the duration as
10 well as the cost of hospitalization.

11 The concept of local, sustained release of antibiotics into infected bone
12 is described in recent literature wherein antibiotic-impregnated PMMA
13 macrobeads are used to treat chronic osteomyelitis. The technique as currently
14 used involves mixing gentamicin with methylmethacrylate bone cement and
15 molding the mixture into beads that are 7mm in diameter. These beads are
16 then locally implanted in the infected site at the time of surgical debridement to
17 serve as treatment. There are, however, significant problems with this
18 method. These include: 1) initially, large amounts of antibiotics diffuse from
19 the cement but with time the amount of antibiotic leaving the cement gradually
20 decreases to subtherapeutic levels; 2) the bioactivity of the antibiotic gradually
21 decreases; 3) methylmethacrylate has been shown to decrease the ability of
22 polymorphonuclear leukocytes to phagocytize and kill bacteria; 4) the beads do
23 not biodegrade and usually must be surgically removed; and 5) the exothermic
24 reaction that occurs during curing of methymethacrylate limits the method to

1 the incorporation of only thermostable antibiotics (primarily aminoglycosides).
2 Nevertheless, preliminary clinical trials using these beads indicate that they are
3 equivalent in efficacy to longer term (4-6 weeks) administration of systemic
4 antibiotics.

5 In many instances, infectious agents have their first contact with the
6 host at a mucosal surface; therefore, mucosal protective immune mechanisms
7 are of primary importance in preventing these agents from colonizing or
8 penetrating the mucosal surface. Numerous studies have demonstrated that a
9 protective mucosal immune response can best be initiated by introduction of
10 the antigen at the mucosal surface, and parenteral immunization is not an
11 effective method to induce mucosal immunity. Antigen taken up by the gut-
12 associated lymphoid tissue (GALT), primarily by the Peyer's patches in mice,
13 stimulates T helper cell (Th) to assist in IgA B cell responses or stimulates T
14 suppressor cells (Ts) to mediate the unresponsiveness of oral tolerance.
15 Particulate antigen appears to shift the response towards the (Th) whereas
16 soluble antigens favor a response by the (Ts). Although studies have
17 demonstrated that oral immunization does induce an intestinal mucosal immune
18 response, large doses of antigen are usually required to achieve sufficient local
19 concentrations in the Peyer's patches. Unprotected protein antigens may be
20 degraded or may complex with secretory IgA in the intestinal lumen.

21 In the process of vaccination, medical science uses the body's innate
22 ability to protect itself against invading agents by immunizing the body with
23 antigens that will not cause the disease but will stimulate the formation of
24 antibodies that will protect against the disease. For example, dead organisms

1 are injected to protect against bacterial diseases such as typhoid fever and
2 whooping cough, toxins are injected to protect against viral diseases such as
3 poliomyelitis and measles.

4 It is not always possible, however, to stimulate antibody formation
5 merely by injecting the foreign agent. The vaccine preparation must be
6 immunogenic, that is, it must be able to induce an immune response. Certain
7 agents such as tetanus toxoid are innately immunogenic, and may be
8 administered in vaccines without modification. Other important agents are not
9 immunogenic, however, and must be converted into immunogenic molecules
10 before they can induce an immune response.

11 The immune response is a complex series of reactions that can
12 generally be described as follows:

- 13 1. the antigen enters the body and encounters antigen-presenting cells
14 which process the antigen and retain fragments of the antigen on their surfaces;
- 15 2. the antigen fragment retained on the antigen presenting cells are
16 recognized by T cells that provide help to B cells; and
- 17 3. the B cells are stimulated to proliferate and divide into antibody
18 forming cells that secrete antibody against the antigen.

19 Most antigens only elicit antibodies with assistance from the T cells
20 and, hence, are known as T-dependent (TD). These antigens, such as
21 proteins, can be processed by antigen presenting cells and thus activate T cells
22 in the process described above. Examples of such T-dependent antigens are
23 tetanus and diphtheria toxoids.

1 Some antigens, such as polysaccharides, cannot be properly processed
2 by antigen presenting cells and are not recognized by T cells. These antigens
3 do not require T cell assistance to elicit antibody formation but can activate B
4 cells directly and, hence, are known as T-independent antigens (TI). Such T-
5 independent antigens include H.influenzae type by polyribosyl-ribitol-phosphate
6 and pneumococcal capsular polysaccharides.

7 T-dependent antigens vary from T-independent antigens in a number of
8 ways. Most notably, the antigens vary in their need for an adjuvant, a
9 compound that will nonspecifically enhance the immune response. The vast
10 majority of soluble T-dependent antigens elicit only low level antibody
11 responses unless they are administered with an adjuvant. It is for this reason
12 that the standard DPT vaccine (diphtheria, pertussis, tetanus) is administered
13 with the adjuvant alum. Insolubilization of TD antigens into an aggregated
14 form can also enhance their immunogenicity, even in the absence of an
15 adjuvant. Golub ES and WO Weigle, J. Immunol. 102:389, 1969). In
16 contrast, T-independent antigens can stimulate antibody responses when
17 administered in the absence of an adjuvant, but the response is generally of
18 lower magnitude and shorter duration.

19 Four other differences between T-independent and T-dependent antigens
20 are:

21 a) T-dependent antigens can prime an immune response so that a
22 memory response can be elicited upon secondary challenge with the same
23 antigen. Memory or secondary responses are stimulated very rapidly and
24 attain significantly higher titers of antibody that are seen in primary responses.

1 T-independent antigens are unable to prime the immune system for secondary
2 responsiveness.

3 b) The affinity of the antibody for antigen increases with time
4 after immunization with T-dependent but not T-independent antigens.

5 c) T-dependent antigens stimulate an immature or neonatal
6 immune system more effectively than T-independent antigens.

7 d) T-dependent antigens usually stimulate IgM, IgG1, IgG2a, and
8 IgE antibodies, while T-independent antigens stimulate IgM, IgG1, IgG2b, and
9 IgG3 antibodies.

10 These characteristics of T-dependent vs. T-independent antigens provide
11 both distinct advantages and disadvantages in their use as effective vaccines.

12 T-dependent antigens can stimulate primary and secondary responses which are
13 long-lived in both adult and in neonatal immune systems, but must frequently
14 be administered with adjuvants. Thus, vaccines have been prepared using only
15 an antigen, such as diphtheria or tetanus toxoid, but such vaccines may require
16 the use of adjuvants, such as alum for stimulating optima responses.

17 Adjuvants are often associated with toxicity and have been shown to
18 nonspecifically stimulate the immune system, thus inducing antibodies of
19 specificities that may be undesirable.

20 Another disadvantage associated with T-dependent antigens is that very
21 small proteins such as peptides, are rarely immunogenic, even when
22 administered with adjuvants. This is especially unfortunate because many
23 synthetic peptides are available today that have been carefully synthesized to

1 represent the primary antigenic determinants of various pathogens, and would
2 otherwise make very specific and highly effective vaccines.

3 In contrast, T-independent antigens, such as polysaccharides, are able
4 to stimulate immune responses in the absence of adjuvants. Unfortunately,
5 however, such T-independent antigens cannot stimulate high level or prolonged
6 antibody responses. An even greater disadvantage is their inability to stimulate
7 an immature or B cell defective immune system (Mond J.J., Immunological
8 Reviews 64:99, 1982) Mosier DE, et al., J. Immunol. 119:1874, 1977).
9 Thus, the immune response to both T-independent and T-dependent antigens is
10 not satisfactory for many applications.

11 With respect to T-independent antigens, it is critical to provide
12 protective immunity against such antigens to children, especially against
13 polysaccharides such as H. influenzae and S. pneumoniae. With respect to T-
14 dependent antigens, it is critical to develop vaccines based on synthetic
15 peptides that represent the primary antigenic determinants of various
16 pathogens.

17 One approach to enhance the immune response to T-independent
18 antigens involves conjugating polysaccharides such H. influenzae PRP (Cruse
19 J.M., Lewis R.E. Jr. ed., Conjugate vaccines in Contributions to Microbiology
20 and Immunology, vol. 10, 1989) or oligosaccharide antigens (Anderson PW, et
21 al., J. Immunol. 142:2464, 1989) to a single T-dependent antigen such as
22 tetanus or diphtheria toxoid. Recruitment of T cell help in this way has been
23 shown to provide enhanced immunity to many infants that have been
24 immunized. Unfortunately, only low level antibody titers are elicited, and

1 only some infants respond to initial immunizations. Thus, several
2 immunizationa are required and protective immunity is often delayed for
3 months. Moreover, multiple visits to receive immunizations may also be
4 difficult for families that live distant from medical facilities (especially in
5 underdeveloped countries). Finally, babies less than 2 months of age may
6 mount little or no antibody response even after repeated immunization.

7 One possible approach to overcoming these problems is to
8 homogeneously disperse the antigen of interest within the polymeric matrix of
9 appropriately sized biodegradable-biocompatible microspheres that are
10 specifically taken up by GALT. Eldridge et al. have used a murine model to
11 show that orally-administered 1-10 micrometer microspheres consisting of
12 polymerized lactide and glycolide, (the same materials used in resorable
13 sutures), were readily taken up into Peyer's patches, and the 1-5 micrometer
14 size were rapidly phagocytized by macrophages. Microspheres that were 5-10
15 micrometers (microns) remained in the Peyer's patch for up to 35 days,
16 whereas those less than 5 micrometer disseminated to the mesenteric lymph
17 node (MLN) and spleen within migrating MAC-1+ cells. Moreover, the
18 levels of specific serum and secretory antibody to staphylococcal enterotoxin B
19 toxoid and inactivated influenza A virus were enhanced and remained elevated
20 longer in animals which were immunized orally with microencapsulated
21 antigen as compared to animals which received equal doses of non-
22 encapsulated antigen. These data indicate that microencapsulation of an
23 antigen given orally may enhance the mucosal immune response against enteric
24 pathogens. AF/R1 pili mediate the species-specific binding of E. coli RDEC-1

1 with mucosal glycoproteins in the small intestine of rabbits and are therefore
2 an important virulence factor. Although AF/R1 pili are not essential for E.
3 coli RDEC-1 to produce enteropathogenic disease, expression of AF/R1 to
4 produce enteropathogenic disease, expression of AF/R1 promotes a more
5 severe disease. Anti-AF/R1 antibodies have been shown to inhibit the
6 attachment of RDEC-1 to the intestinal mucosa and prevent RDEC-1 disease in
7 rabbits. The amino acid sequence of the AF/R1 pilin subunit has recently been
8 determined, but specific antigenic determinants within AF/R1 have not been
9 identified.

10 In the current study we have used these theoretical criteria to predict
11 probable T or B cell epitopes from the amino acid sequence of AF/R1. Four
12 different 16 amino acid peptides that include the predicted epitopes have been
13 synthesized: AF/R1 40-55 as a B cell epitope, 79-94 as a T cell epitope, 108-
14 123 as a T and B cell epitope, and AF/R1 40-47/79-86 as a hybrid of the first
15 eight amino acids from the predicted B cell epitope and the T cell epitope. We
16 have used these peptides as well as the native protein to stimulate the in vitro
17 proliferation of lymphocytes taken from the Peyer's patch, MLN, and spleen
18 of rabbits which have received intraduodenal priming with microencapsulated
19 or non-encapsulated AF/R1. Our results demonstrate the microencapsulation
20 of AF/R1 potentiates the cellular immune response at the level of the Peyer's
21 patch, thus enhancing in vitro lymphocyte proliferation to both the native
22 protein and its linear peptide antigens. CFA/I pili, rigid thread-like structures
23 which are composed of repeating pilin subunits of 147 amino acid found on
24 serogroups 015, 025, 078, and 0128 of enterotoxigenic E. coli (ETEC) (1-4,

1 18). CFA/I promotes mannose resistant attachment to human brush borders
2 (5); therefore, a vaccine that established immunity against this protein may
3 prevent the attachment to host tissues and subsequent disease. In addition,
4 because the CFA/I subunit shares N-terminal amino acid sequence homology
5 with CS1, CFA/II (CS2) and CFA/IV (CS4) (4), a subunit vaccine which
6 contained epitopes from this area of the molecule may protect against infection
7 with various ETEC.

8 Until recently, experiments to identify these epitopes were time
9 consuming and costly; however, technology is now available which allows one
10 to simultaneously identify all the T cell and B cell epitopes in the protein of
11 interest. Multiple Peptide synthesis (Pepscan) is a technique for the
12 simultaneous synthesis of hundreds of peptides on polyethylene rods (6). We
13 have used this method to synthesize all the 140 possible overlapping
14 actapeptides of the CFA/I protein. The peptides, still on the rods, can be used
15 directly in ELISA assays to map B cell epitopes (6, 12-14). We have also
16 synthesized all the 138 possible overlapping decapeptides of the CFA/I protein.
17 For analysis of T cell epitopes, these peptides can be cleaved from the rods
18 and used in proliferation assays (15). Thus this technology allows efficient
19 mapping and localization of both B cell and T cell epitopes to a resolution of a
20 single amino acid (16). These studies were designed to identify antigenic
21 epitopes of ETEC which may be employed in the construction of an effective
22 subunit vaccine.

23 CFA/I pili consist of repeating pilin protein subunits found on several
24 serogroups of enterotoxigenic E coli (ETEC) which promote attachment to

1 human intestinal mucosa. We wished to identify areas within the CFA/I
2 molecule that contain immunodominant T cell epitopes that are capable of
3 stimulating the cell-mediated portion of the immune response in primates as
4 well as immunodominant B cell epitopes. To do this, we (a) resolved the
5 discrepancy in the literature on the complete amino acid sequence of CFA/I,
6 (b) immunized three Rhesus monkeys with multiple i.m. injections of purified
7 CFA/I subunit in Freund's adjuvant, (c) synthesized 138 overlapping
8 decapeptides which represented the entire CFA/I protein using the Pepscan
9 technique (Cambridge Research Biochemicals), (d) tested each of the peptides
10 for their ability to stimulate the spleen cells from the immunized monkeys in a
11 proliferative assay (e) synthesized 140 overlapping octapeptides which
12 represented the entire CFA/I protein, and (f) tested serum from each monkey
13 for its ability to recognize the octapeptides in a modified ELISA assay. A total
14 of 39 different CFA/I decapeptides supported a significant proliferative
15 response with the majority of the responses occurring within distinct regions of
16 the protein (peptides beginning with residues 8-40, 70-80, and 126-137).
17 Nineteen of the responsive peptides contained a serine residue at positions 2,
18 3, or 4 in the peptide, and a nine contained a serine specifically at position 3.
19 Most were predicted to be configured as an alpha helix and have a high
20 amphipathic index. Eight B cell epitopes were identified at positions 3-11, 11-
21 21, 22-29, 32-40, 38-45, 66-74, 93-101, and 124-136. The epitope at position
22 11-21 was strongly recognized by all three individual monkeys, while the
23 epitopes at 93-101, 124-136, 66-74, and 22-29 were recognized by two of the
24 three monkeys.

Recent advances in the understanding of B cell and T cell epitopes have improved the ability to select probably linear epitopes from the amino acid sequence using theoretical criteria. B cell epitopes are often composed of a string of hydrophilic amino acids with a high flexibility index and a high probability of turns within the peptide structure. Prediction of T cell epitopes are based on the Rothbard method which identifies common sequence patterns that are common to known T cell epitopes or the method of Berzofsky and others which uses a correlation between algorithms predicting amphipathic helices and T cell epitopes.

V. SUMMARY OF THE INVENTION

This invention relates to active core materials such as biologically active agent(s), drug(s), or substance(s) encapsulated within a biodegradable-biocompatible polymeric matrix. In view of the enormous scope of this invention it will be presented herein as Phases I, II, and III. Phase I illustrates the encapsulation of antibiotics within a biodegradable-biocompatible polymeric matrix for the prevention and treatment of wound infections. Phase II illustrates the encapsulation of antigens (more specifically, oral-intestinal vaccine antigens) within a biodegradable-biocompatible polymeric matrix against diseases such as those caused by enteropathogenic organism. Phase III illustrates the use of a biodegradable-biocompatible polymeric matrix for burst-free programmable sustained release of biologically active agents, inclusive of peptides, over a period of up to 100 days in an aqueous physiological environment.

1 Controlled drug delivery from a biodegradable-biocompatible matrix
2 offers profound advantages over conventional drug/antigen dosing.
3 Drugs/antigens can be used more effectively and efficiently, less drug/antigen
4 is required for optimal therapeutic effect and, in the case of drugs, toxic side
5 effects can be significantly, reduced or essentially eliminated through drug
6 targeting. The stability of some drugs/antigens can be improved allowing for a
7 longer shelf-life, and drugs/antigens with a short half-life can be protected
8 within the matrix from destruction, thereby ensuring sustained release of active
9 agent over time. The benefit of a continuous sustained release of drug/antigen
10 is beneficial because drug levels can be maintained within a constant
11 therapeutic range and antigen can be presented either continuously or in a
12 pulsatile mode as required to stimulate the optimal immune response. All of
13 this can be accomplished with a single dose of encapsulated drug/antigen.

14 This invention contemplates, but is not limited to, medically acceptable
15 methods for the effective local delivery of biologically active agents that, of
16 themselves, are directly (e.g. drugs, such as antibiotics) or indirectly (e.g.
17 vaccine antigens) therapeutic or prophylactic. It also includes drugs/agents that
18 elicit/modulate natural biological activity.

19 Wounds characterized by the presence of infection, devitalized tissue,
20 and foreign-body contaminants have high infection rates and are difficult to
21 treat. This invention describes antibiotic formulation encapsulated within
22 microspheres of a biodegradable-biocompatible polymer that, when applied
23 locally to contaminated or infected wounds, provides immediate, direct, and
24 sustained (over a period up to 100 days), high concentrations of antibiotic in

1 the wound site (soft tissue and bone). By encapsulating antibiotics and
2 applying them directly, one can achieve a significant reduction in nonspecific
3 binding of the drug to body proteins, a phenomena commonly observed
4 following conventional systemic administration of free drugs. Thus, less drug
5 is required, higher concentrations are maintained at the site of need, and
6 efficacy is enhanced. This approach provides superior treatment over
7 conventional systemic administration of antibiotics for wound infections
8 because higher bacteriocidal concentrations can be achieved and maintained in
9 the wound environment. Higher concentrations kill more bacteria.

10 Applicants' invention for this application is described in Phase I.

11 Furthermore, applicants reasoned that a protective mucosal immune response
12 might be best initiated by introduction of an antigen at the mucosal surface,
13 because unprotected protein antigens delivered in a free form may be degraded
14 or may complex with secretory IgA in the intestinal lumen precluding entry
15 and subsequent processing in local immune cells. The formulation of
16 microspheres containing antigen small enough in size to be phagocytized
17 locally in the gut was envisioned as being able to induce an elevated localized
18 immune response. Applicants' invention for this application is described in
19 Phase II. In summary, applicants propose using several methods for the local
20 application of drugs including: 1) the direct application of the encapsulated
21 drug to a surgical/traumatized area, 2) oral delivery that provides either local
22 deposition of microencapsulated antigen/drugs at mucosal membranes or
23 transport across these membranes to provide local adherence of
24 microencapsulated drugs/antigen to mucosal membranes to provide sustained

1 release of drug/antigen into soft tissue or a body cavity, and/or 3) sustained
2 intercellular or extracellular drug/antigen release following subcutaneous
3 injection.

4 In those instances where antibiotics are administered locally, applicants
5 have found that the controlled release of the antibiotic from within a
6 biodegradable-biocompatible polymeric matrix within 14 days to about 4
7 weeks without significant drug trailing is especially useful. However, if
8 desired, the release of a biologically active agent from a polymeric matrix
9 comprised of an active agent and a blend of uncapped and end-capped
10 biodegradable poly DL(lactide-co-glycolide), can be controlled over a period of
11 1 to about 100 days without significant drug dumping or trailing. Such novel
12 biocompatible-biodegradable microspheres developed with a burst-free
13 programmable sustained release of biologically active agents, inclusive of
14 polypeptides, are described in applicants' U.S. Patent Application Serial No.
15 08/590,973 filed January 24, 1996.

16 When antibiotics are administered systemically in the conventional
17 manner, or locally as contemplated by the applicants, the immune response to
18 the antibiotic and the potential for hypersensitivity and/or anaphylactoid
19 response (especially to beta-lactam antibiotics such as penicillins/ampicillin) is
20 a clinical concern. In early studies the inventors observed a specific IgG
21 response to ampicillin as it was released from the microencapsulated
22 formulation (illustrated in the histogram, Figure 1 and 2). This response is
23 reminiscent of antibody elicited by vaccine antigens in conventional vaccines.
24 The response to vaccine antigens is known to be accentuated by the use of an

1 adjuvant such as alum. Alum is a crude, less adaptable delivery vehicle than
2 its counterpart, the biodegradable-biocompatible poly DL(lactide-co-glycolide),
3 of this invention - the polymeric matrix. This knowledge stimulated additional
4 studies relevant to the effects of sustain release of agents on the immune
5 response.

6 There are, in general, two forms of localized delivery which can be
7 achieved with PLGA microspheres-delivery which is localized to individual
8 cells of the body (intracellular delivery); and delivery which is localized to
9 tissues within a specific region of the body (localized extracellular delivery).

10 Applicants have prepared antibiotic and hepatitis vaccine formulations
11 which functioned by delivering localized extracellular doses of their active
12 agents. This was achieved by using relatively large microspheres which served
13 as a depot for the drug or antigen. Their large size 40-100 microns in diameter
14 precluded their being phagocytized or diffusing throughout the intercellular
15 fluid compartments of the body. Their drug agent loads were thus released
16 within their immediate vicinity which resulted in the generation of very high
17 local concentrations of antibiotic or the release of sufficiently high
18 concentrations of free antigen to induce an immune response.

19 The large-diameter antibiotic bearing microspheres were originally
20 developed by applicants primarily for topical application on exposed debrided
21 tissues of combat wounds. However, an inherent property exhibited by the
22 antibiotics when topically applied to a wound site is the generation of
23 measurable levels of immune response. This concept of local delivery by

1 topical application of microspheres to tissue to achieve localized concentrations
2 of therapeutic agents was subsequently applied to the development of an oral
3 vaccine for protection against traveler's diarrhea caused by E. coli. Vaccine
4 antigen was encapsulated into microspheres whose diameters were
5 predominantly in the 5-10 micron size range based on an understanding that
6 microspheres of this size would not readily be either phagocytized or
7 transported across the gut wall into the body. Ingestion of these microspheres
8 thus constituted a localized delivery achieved by topical application of the
9 spheres to the wall tissue of the gut. This topical application resulted in the
10 localized trapping of a small percentage of these sphere into the Peyer's
11 patches where the spheres proceeded to release their antigen in a localized
12 fashion to immune cells located within the intestinal Patches.

13 The concept of localized sustained local delivery has been further
14 extended to the delivery of analgesics and anesthetics to exposed dental pulp to
15 control pain and inflammatory responses. Again, the PLGA microsphere used
16 for this type of delivery are relatively large (40-100 um in diameter) and serve
17 as a topical depot for localized extracellular release of the drug.

18 Consistent with their understanding of the inherent immunogenic
19 properties exhibited by active core materials in vivo, applicants have moved on
20 to other non-topical application methods of using their microsphere delivery
21 system. Some of these center on the use of small diameter microspheres
22 ranging from sub micron to under 5 microns in diameter. These spheres allow
23 intracellular targeting of drug or antigen. They also allow for transmucosal
24 delivery of drugs or antigens. The concept of localized delivery in these

1 instances refers to the localized delivery of drug or agent within individual
2 target cells of the body regardless of their location or distribution within the
3 body. This approach is useful in development of antitubercular, antimalarial,
4 antiviral, and antichlamydial formulations against intracellular parasites. It is
5 also useful for the development of vaccines against intracellular parasites and
6 for direct delivery of agents to presenting cells of the immune system.

7 Another nontopical application method of using PLGA microspheres
8 resides in their usefulness as injectable depots for drugs intended for either
9 localized or systemic delivery. Typically larger diameter microspheres are
10 used for depots as these are less likely to diffuse away. The local or systemic
11 nature of these delivery systems is, in part a function of the release rate of the
12 drug from the depot and the diffusional and solubility characteristics of the
13 drug being released. Cancer chemotherapeutics, systemic antibiotics, delivery
14 of antibiotics to infected bone are potential application of this system.
15 Additional this non-topical systemic depot application can be extended to the iv
16 injection of cancer-agent laden microspheres to embolize and destroy a
17 malignant tumor. Additionally, the PLGA microspheres can be used as a
18 carrier to deliver substances useful for the in modification of cells or genes in
19 bioengineering or genetic procedures.

20 Interest in the concept that antigens encapsulated within a
21 biodegradable-biocompatible polymeric matrix could be formulated as a
22 vaccine with superior efficacy over conventional vaccines, originated from the
23 inventors' own observations that the drug, ampicillin, when sustain released
24 from poly DL(lactide-co-glycolide) elicited antibody production. In these

1 studies, the applicants were able to measure specific IgG antibodies to free
2 ampicillin and to ampicillin released from microencapsulated ampicillin
3 formulations in the sera of mice previously "treated" with the ampicillin
4 formulations using ELISA. Numerous other studies also document the ability
5 of beta-lactam antibiotic to elicit antibody. Selected, more recent studies
6 whose findings are consistent with earlier discoveries made by applicants when
7 conducting experiments with ampicillin include those by Klein et al. (1993)
8 who detected specific IgG antibodies (IgG and IgG3 subclasses) to the B-
9 lactam ring in patients receiving penicillin therapy, work by Nagakura et al.
10 (1990) which detected specific antibodies to cephalexin, a B-lactam antibiotic
11 in the sera of guinea pigs, and Auci et al. (1993) who detected benzyl
12 penicilloyl specific IgM, IgG IgE, and IgA antibody forming cells in lymphoid
13 cells of mice given benzyl penicilloyl-Keyhole Limpet Hemocyanin.
14 Pharmaceutical compositions of antigens encapsulated with poly DL(lactide-co-
15 glycolide) are described in Phase II. The microspheres of the invention allow
16 for introduction of vaccine antigens to mucosal surfaces in particles that can be
17 subsequently taken up locally by phagocytic cells. Such an approach for both
18 drugs and antigens provides significant advantages in potency and efficacy over
19 conventional systemically administered drugs or vaccines. A partial list of
20 biologically active agents or drugs that will potentially derive significant
21 medical benefits from this delivery system includes: antibacterial agents;
22 peptides; polypeptides; antibacterial peptides; antimycobacterial agents;
23 antimycotic agents; antiviral agents; antiparasitic agents;, antifungal; antiyeast
24 agents; hormonal peptides; cardiovascular agents; hormonal

1 peptides; cardiovascular agents; narcotic antagonists; analgesics; anesthetics;
2 insulins; steroids including HIV therapeutic drugs (including protease
3 inhibitors) and AZT; estrogens; progestins; gastrointestinal therapeutic agents;
4 non-steroidal anti-inflammatory agents; parasympathoimetic agents;
5 psychotherapeutic agents; tranquilizers; decongestants; sedative-hypnotics; non-
6 estrogenic and non-progestional steroids; sympathomimetic agents; vaccines;
7 vitamins; nutrients; anti-migraine drugs; electrolyte replacements; ergot
8 alkaloids; anti-inflammatory agents; prostaglandins; cytotoxic drugs; antigens;
9 antibodies; enzymes; growth factors; immunomodulators; pheromones;
10 prodrugs; psychotropic drugs; nicotine; antiblood clotting drugs; appetite
11 suppressants/stimulants and combinations thereof; contraceptive agents include
12 estrogens such as diethyl silbestrol; 17-beta-estradiol; estrone; ethinyl estradiol;
13 mestranol; progestins such as norethindrone; norgestryl; ethynodiol diacetate;
14 lynestrenol; medroxyprogesterone acetate; dimethisterone; megestrol acetate;
15 chlormadinone acetate; norgestimate; norethisterone; ethisterone; melentate;
16 norgestimate; norethisterone; ethisterone; melengestrol; norethynodrel; and
17 spermicidal compounds such as nonyphenoxypolyoxyethylene glycol;
18 benzethonium chloride; chlorindanol; include gastrointestinal therapeutic agents
19 such as aluminum hydroxide; calcium carbonate; magnesium carbonate;
20 sodium carbonate and the like; non-steroidal antifertility agents;
21 parasympathomimetic agents; psychotherapeutic agents; major tranquilizers
22 such as chloropromazine HCL; clozapine; mesoridazine; metiapine;
23 reserpine; thioridazine; minor tranquilizers such as chlordiazepoxide;
24 diazepam; meprobamate; temazepam and the like; rhinological decongestants;

1 sedative-hypnotics such as codeine; phenobarbital; sodium pentobarbital;
2 sodium secobarbital; other steroids such as testosterone and testosterone
3 propionate; sulfonmides; sympathomimetic agents; vaccines; vitamins and
4 nutrient such as the essential amino acids; essential fats; anti-HIV agents;
5 including AZT; antimalarials such as 4-aminoquinolines; 8 aminoquinolines;
6 pyrimethamine; anti-migraine agents such as mazindol; phentermine; anti-
7 Parkinson agents such as L-dopa; antispasmodics such as atropine;
8 methscopolamine bromide; antispasmodics and anticholinergic agents such as
9 bile therapy; digestants; enzymes and the like; antitussives such as
10 dextromethorphan and noscapine; bronchodilators; cardiovascular agents such
11 as anti-hypertensive compounds; Rauwolfia alkaloids; coronary vasodilators;
12 nitroglycerin; organic nitrites; pentaerythritetranitrate; electrolyte
13 replacements such as potassium chloride; ergotalkaloids such as ergotamine
14 with and without caffeine; hydrogenated ergot alkaloids; dihydroergocristine
15 methanesulfate; dihydroergocornine methanesulfonate; dihydroergokroptine
16 methaneusulfate and combinations thereof; alkaloids such as atropine sulfate;
17 Belladonna; hyoscine hydrobromide; analgesics; narcotics such as codeine;
18 dihydrocodienone; meperidine; morphine; non-narcotics such as salicylates;
19 aspirin; acetaminophen; and d-propoxyphene; antibiotics such as the
20 cephalosporins including ceflacor and cefuroxime; chloranphenical; gentamicin;
21 Kanamycin A. Kanamycin B; the penicillins; ampicillin; amoxicillin;
22 streptomycin A; antimycin A; chloropamtheniol; metromidazole;
23 oxytetracycline penicillin G; the tetracyclines; including minocycline; fluoro-
24 quinolones including ciprofloxacin; ofloxacin; macrolides including

1 clarithromycin; erythromycin; aminoglycosides including gentamicin;
2 amikacin; tobramycin and kanamycin; beta-lactams including ampicillin;
3 polymyxin-B; amphotericin-B; aztreonam; chloramphenicol; fusidic;
4 lincosamides; metronidazole; nitro-furantoin; imipenem/cilastatin; quinolones;
5 systemic antibiotics including rifampin; polymyxins; sulfonamides; trimethoprim;
6 glycopeptides including vancomycin; teicoplanin and imidazoles; anti-cancer
7 agents; including anti-Kaposi's sarcoma; anti-convulsants such as mephenytoin;
8 phenobarbital; trimethadione; anti-emetics such as triethylperazine;
9 antihistamines such as chlorphenirazine; dimenhydrinate; diphenhydramine;
10 perphenazine; triprolidine and the like; anti-inflammatory agents such as
11 hormonal agents; hydrocortisone; prednisolone; prednisone; non-hormonal
12 agents; allopurinol; for claims water-soluble hormone drugs; antibiotics;
13 antitumor agents; anti-inflammatory agents; antipyretics; analgesics;
14 antitussives; expectorants; sedatives; muscle relaxants; antiepileptics; anticancer
15 agents; antidepressants; antiallergic drugs; cardiotonics; antiarrhythmic drugs;
16 vasodilators; antihypertensives; diuretics; anticoagulants; and antinarcotics; in
17 the molecular weight range of 100-100,000 daltons; indomethacin;
18 phenylbutazone; prostaglandins; cytotoxic drugs such as thiopeta; chloramucil;
19 cyclophosphamide; melphalan; nitrogen mustard; methotrexate; antigens such as
20 proteins; glycoproteins; synthetic peptides; carbohydrates; synthetic
21 polysaccharides; lipids; glycolipids; lipopolysaccharides(LPS); synthetic
22 lipopolysaccharides and with or without attached adjuvants such as synthetic
23 muramyl dipeptide derivatives; antigens of such microorganisms as Neisseria
24 gonorrhoea; Mycobacterium tuberculosis; P. carinii Pneumonia; Herpes virus

(humonis types 1 and 2); Herpes zoster; Candidia albicans; Candida tropicalis; Trichomonas vaginalis; Haemophilus vaginalis; Group B streptococcus ecoli; Microplasma hominis; Hemophilus ducreyi; Granuloma inguinale; Lymphopathia venerum; Treponema palidum; Brucela aborus Brucela meitensis Brucela suis; Brucella canis Campylobacter fetus; Campylobacer fetus intesinalis; Leptospira pomona. Listeria monocytogenes; Brucella ovis; Equine herpes virus 1; Equine arteritis virus; IBR-IBP virus; Chlamydia psittaci; Trichomonas foetus; Taxoplasma gondii; Escherichia coli; Actinobacillus equuli; Salmonella abortus ovis. Salmonella abortus eui; Pseudomonas aeruginosa; Corynebacterium equi; Corynebacterium pyogenes; Actinobaccilus seminis; Mycoplasma bovigenitalium; Aspergillus fumigatus; Absidia ramosa; Trypanosoma equiperdum; Babesia cabali; Clostridium tetani; antibodies which counteract the above microorganisms; and enzymes such as ribonuclease; neuramidinase; trypsin; glycogen phosphorylase; sperm lactic dehydrogenase; sperm hyaluronidase; adenossinetriphosphase; alkaline phosphatase; alkaline phospho esterase; amino peptides; tpsin chymotrypsin amylase; muramidase; acrosomal proteinase; diesterase; glutamic acid dehydrogense; succunic and dehydrogenase; beta-glycophosphatase lipase; ATP-ase alpha-peptate gamma-glutamiotranspeptidase; sterold-beta-ol-dehydrogenase; DPN-di-aprorase; and combinations thereof. Having generally described the invention; a further understanding can be obtained by reference to certain specific examples which are provided herein for purpose of illustration only and are not intended to be limiting unless otherwise specified. Moreover; the polymeric matrix of this invention may be used for the in situ production and controlled release of

1 products that are produced by the controlled release of encapsulated reactants.
2 Additionally; effective testing or monitoring devices for chemical agents or
3 bioactive agents can be made by encapsulating reagents which react as they are
4 released from the polymeric matrix, with agents sought to be detected. The
5 novel delivery system of this invention is applicable to all categories of active
6 substances capable of being used for the prevention and/or treatment of human,
7 animal and plant diseases. This delivery system is also applicable to the
8 design of novel diagnostic tests. Additionally, it can be useful for the delivery
9 to a subject of a polyfunctional mixture or cocktail formulation of encapsulated
10 active (i.e. biologically) substances for the prevention and/or treatment of
11 diseases the same or different. The encapsulated formulation ingredients
12 would be comprised of multiple drugs, multiple vaccines or a combination
13 thereof.

14 Applicants contemplate that the invention will be useful in the formul-
15 ation of disease specific compositions for the prevention and/or treatment of
16 diseases and/or ailments which include: viral infections; bacterial infections;
17 fungal infections; yeast infections; parasitic infections and more specific
18 diseases and/or ailments; such as as, aids; alzheimer's dementia; angiogenesis
19 diseases; aphthour ulcers in AIDS patients; asthma; atopic dermatitis;
20 psoriasis; basal cell carcinoma; benign prostatic hypertrophy; blood substitute;
21 blood substitute in surgery patients; blood substitute in trauma patients; breast
22 cancer; breast cancer; cutaneous & metastatic; cachexia in AIDS;
23 campylobacter infection; cancer; pneumonia; sexually transmitted diseases
24 (STDs); cancer; viral diseases; candida albicans in AIDS and cancer;
25 candidiasis in HIV infection; pain in

1 cancer; pancreatic cancer; parkinson's disease; peritumoral brain edema;
2 postoperative adhesions (prevent); proliferative diseases; prostate cancer;
3 ragweed allergy; renal disease; restenosis; rheumatoid arthritis; rheumatoid
4 arthritis; allergies; rotavirus infection; scalp psoriasis; septic shock; small-cell
5 lung cancer; solid tumors; stroke; thrombosis; type I diabetes; type I diabetes
6 w/kidney transplants; type II diabetes; visceral leishmaniasis; malaria;
7 periodontal or gum disease; cardiac rhythm disorders; central nervous system
8 diseases; central nervous system disorders; cervical dystonia (spasmodic
9 torticollis); choroidal neovascularization; chronic hepatitis c, b and a; colitis
10 associated with antibiotics; colorectal cancer; coronary artery thrombosis;
11 cryptosporidiosis in AIDS; cryptosporidium parvum diarrhea in AIDS; cystic
12 fibrosis; cytomegalovirus disease; depression; social phobias; panic disorder;
13 diabetic complications; diabetic eye disease; diarrhea associated with
14 antibiotics; erectile dysfunction; genital herpes; graft-vs host disease in
15 transplant patients; growth hormone deficiency; head and neck cancer; head
16 trauma; stroke; heparin neutralization after cardiac bypass; hepatocellular
17 carcinoma; HIV; HIV infection; huntington's disease; CNS diseases;
18 hypercholesterolemia; hypertension; inflammation; inflammation and
19 angiogenesis; inflammation in cardiopulmonary bypass; influenza; migraine head
20 ache; interstitial cystitis; kaposi's sarcoma; kaposi's sarcoma in AIDS; lung
21 cancer; melanoma; molluscum contagiosum in AIDS; multiple sclerosis;
22 neoplastic meningitis from solid tumors; non-small cell lung cancer; organ
23 transplant rejection; osteoarthritis; rheumatoid arthritis; osteoporosis; drug
24 addiction; shock; ovarian cancer; and pain.

1 Also contemplated here are those diseases or health conditions capable
2 being benefitted by the list of biologically active agents or drugs previously
3 listed in the Summary of the Invention.

4 EFFECTS OF MICROENCAPSULATED ANTIBIOTICS ON THE IMMUNE
5 RESPONSE

6 Preclinical studies evaluating microencapsulated antibiotics in animals
7 have demonstrated that targeted local release of antibiotics directly into
8 infected soft tissue and bone via sustained release of the drug from poly
9 DL(lactide-co-glycolide) will greatly enhance antibiotic efficacy for both
10 prophylaxis and treatment. Antibiotic hypersensitivity was, from the
11 beginning, the most obvious untoward clinical concern of this novel approach
12 to antibiotic delivery. What effect would sustained antibiotic release have on
13 the hypersensitive patient?

14 Prior to the filing of applicants' parent application Serial No. 590,308
15 on March 16, 1984, which disclosed the local application of encapsulated
16 antibiotics to treat wound infection, it was commonly known that an inherent
17 property of free antibiotics such as ampicillin, is that they elicit an immune
18 response in man and induce the production of antibodies. Thus, interest in the
19 immune response elicited from the sustained release of immunogens intensified
20 in order to capture the beneficial aspects of this event immunogenic event in a
21 manner which would advance the frontiers of medical science. This led to
22 additional studies with sustain released antibiotics and led the inventors to
23 postulate that antigens encapsulated in lactide/glycolide could potentially

1 provide a more effective method of active immunization than free antigen
2 alone. In follow on experiments, vaccine antigens were encapsulated and
3 studies were performed to explore this hypothesis as illustrated in Phase II,
4 herein (Phase II).

5 VI. BRIEF DESCRIPTION OF THE DRAWINGS

6 Figure 1 shows the effect of microencapsulated ampicillin (MEAA) on
7 the immune response when mice are treated with free ampicillin, ampicillin
8 encapsulated within biodegradable-biocompatible microspheres and placebo
9 poly (Lactide/glycolide) microspheres, by measuring the specific IgG
10 antibodies to free ampicillin and MEAA in sera of treated mice by ELISA.

11 Figure 2 shows that guinea pigs sensitized with free or
12 microencapsulated ampicillin developed specific IgG antibodies to ampicillin as
13 measured by ELISA.

Figure 3 shows the in vitro release of [^{14}C]-ampicillin anhydrate from sterilized microcapsules/spheres (45 to 106 micrometers in diameter) into 0.1 molar potassium phosphate receiving fluid (pH 7.4) maintained at 37°C. The microcapsules consisted of about 10 weight percent ampicillin anhydrate and about 65 weight percent 53:47 DL-PLG polymer.

Figure 4 shows the in vitro release of [^{14}C]-ampicillin anhydrate from sterilized microcapsules (10 to 100 micrometers consisting of about 35 weight percent ampicillin and about 65 weight percent of 53:47 DL-PLG polymer.

Figure 5 shows the mean daily excretion of [^{14}C] from rats receiving subcutaneous injections of sterilized microencapsulated and unencapsulated [^{14}C]-ampicillin anhydrate.

Figure 6 illustrates that encapsulated as well as the ampicillin anhydrate showed a fast release of drug during Day 1. By Day 4, the amount of ampicillin found in the serum of animals dosed with the unencapsulated drug was below the level of detection of the assay, whereas serum levels of ampicillin were detectable in animals receiving encapsulated ampicillin for up to 11 days.

Figure 7 shows mean serum levels of ampicillin at 1-hour following implantation of either microencapsulated ampicillin or

unencapsulated ampicillin into the medullary canal of the rabbit tibia with experimental osteomyelitis.

Serum Cefazolin Levels. Figure 8 shows the mean serum concentrations of cefazolin that were measured at 1 hour and 24 hours following local antibiotic therapy with either CZ microspheres (Group A) or free CZ powder (Group B) in the rabbit fracture-fixation model. At 1 hour, the mean serum cefazolin levels were approximately 32 times higher for the Group B animals who had received local antibiotic therapy with free CZ powder (18.7 ± 6.1 ug/ml) as compared to the Group A animals who were treated with CZ microspheres (0.57 ± 0.27 ug/ml). This difference in the mean serum cefazolin levels between the two groups was statistically significant ($p = 0.0023$) by Student's t test. At 24 hours following local treatment, no cefazolin was detected in the sera of the rabbits who had received free CZ powder (Group B), however, low cefazolin concentrations were detected in the sera of Group A animals who were treated with the CZ microspheres. It is evident from the data that the free antibiotic diffuses rapidly from the wound and is absorbed into the systemic circulation, whereas, the microspheres remain localized and continue to release low but measurable levels of antibiotic for an extended time interval.

Figure 9 shows the size distribution of microspheres wherein the particle size distribution (%) is (a) By number 1-5 (91) and 6-10 (9) and (b) By weight 1-5 (28) and 6-10 (72).

Figure 10 shows a scanning electron micrograph of microspheres.

Figures 11(a) and (b) show the In vitro immunization of spleen cells and demonstrates that AF/RI pilus protein remains immunogenic to rabbit spleen cells immunized in vitro after microencapsulation. AF/RI pilus protein has been found to be immunogenic for rabbit spleen mononuclear cells in vitro producing a primary IgM antibody response specific to AF/RI. Immunization with antigen encapsulated in biodegradable, biocompatible microspheres consisting of lactide/glycolide copolymers has been shown to endow substantially enhanced immunity over immunization with the free antigen. To determine if microencapsulated AF/RI maintains the immunogenicity of the free pilus protein, a primary in vitro immunization assay was conducted. Rabbit spleen mononuclear cells at a concentration of 3×10^5 cells/well. Triplicate wells of cells were immunized with free AF/RI in a dose range from 15 to 150 ng/ml or with equivalent doses of AF/RI contained in microspheres. Supernatants were harvested on days 7, 9, 12, and 14 of culture and were

1 assayed for free AF/RI pilus protein specific IgM antibody by the ELISA.
2 Supernatant control values were subtracted from those of the immunized cells.
3 Cells immunized with free pilus protein showed a significant positive IgM
4 response on all four days of harvest, with the antibody response increasing on
5 day 9, decreasing on day 12, and increasing again on day 14. Cells
6 immunized with microencapsulated pilus protein showed a comparable positive
7 IgM antibody response as cells immunized with free pilus protein. In
8 conclusion, AF/RI maintains immunogenicity to rabbit spleen cells immunized
9 in vitro after microencapsulation.

10 Figures 12(a) and (b) show in vitro immunization of Peyer's
11 patch cells. Here the AF/RI pilus protein remains immunogenic to rabbit
12 Peyer's patch cells immunized in vitro after microencapsulation. AF/RI pilus
13 protein has been found to be immunogenic for rabbit Peyer's patch
14 mononuclear cells in vitro producing a primary IgM antibody response specific
15 to AF/RI. Immunization with antigen encapsulated in biodegradable,
16 biocompatible microspheres consisting of lactide/glycolide copolymers has been
17 shown to endow substantially enhanced immunity over immunization with the
18 free antigen. To determine if microencapsulated AF/RI maintains the
19 immunogenicity of the free pilus protein, a primary in vitro immunization assay
20 was conducted. Rabbit Peyer's patch mononuclear cells at a concentration of
21 3×10^6 cells/ml were cultured in 96-well, round bottom microculture plates at a
22 final concentration of 6×10^5 cells/well. Triplicate wells of cells were
23 immunized with free AF/RI in a dose range from 15 to 150 ng/ml or with
24 equivalent dose of AF/RI contained in microspheres. Supernatants were

1 harvested on days 7, 9, 12, and 14 of culture and were assayed for free
2 AF/RI pilus protein specific IgM antibody by the ELISA. Supernatant control
3 values were subtracted from those of the immunized cells. Cells immunized
4 with free pilus protein showed a significant positive IgM response on all four
5 days of harvest, with the highest antibody response on day 12 with the highest
6 antigen dose. Cells immunized with encapsulated pilus protein showed a
7 positive response on day 12 with all three antigen doses. In conclusion, AF/RI
8 pilus protein maintains immunogenicity to rabbit Peyer's patch cells
9 immunized in vitro after microencapsulation.

10 Figure 13 shows proliferative responses to AF/RI by rabbit
11 Peyer's patch cells. Naive rabbits were primed twice with 50 micrograms of
12 either non-encapsulated (rabbits 132 and 133) or microencapsulated (rabbits
13 134 and 135) AF/RI pili by endoscopic intraduodenal inoculation seven days
14 apart. Seven days following the second priming, Peyer's patch cells were
15 cultured with AF/RI in 96-well plates for four days followed by a terminal six
16 hour pulse with [³H]thymidine. Data shown is the SI calculated from the
17 mean cpm of quadruplicate cultures. Responses were significant for all
18 rabbits: 132 (p=0.013), 133 (p=.0006), 134 (p=0.0016), and 135
19 (p=0.0026). Responses were significantly different between the two groups.
20 Comparison of the best responder in the nonencapsulated antigen group (rabbit
21 133) with the lowest responder in the microencapsulated antigen group (rabbit
22 134) demonstrated an enhanced response when the immunizing antigen was
23 microencapsulated (p=0.0034).

1 Additionally, Figure 13 relates to the in vitro lymphocyte
2 proliferation after sensitization of rabbit lymphoid tissues with encapsulated or
3 non-encapsulated AF/RI pilus adhesion of E. coli strain RDEC-1. The AF/RI
4 adherence factor is a plasmid encoded pilus protein that allows RDEC-1 to
5 attach to rabbit intestinal brush borders. We investigated the
6 immunopotentiating effect of encapsulating purified AF/RI into biodegradable
7 non-reactive microspheres composed of polymerized lactide and glycolide,
8 materials used in resorbable sutures. The microspheres had a size range of
9 5-10 microns, a size selected for Peyer's Patch localization, and contained
10 0.62% protein by weight. NZW rabbits were immunized twice with 50
11 micrograms of either encapsulated or non-encapsulated AF/RI by intraduodenal
12 later of non-encapsulated AF/RI by intraduodenal inoculation seven days apart.
13 Lymphocyte proliferation in response to purified AR/RI was conducted in vitro
14 at seven days and showed that encapsulating the antigen into microspheres
15 enhanced the cellular immune response in the Peyer's Patch; however, no
16 significant increase was observed in spleen or mesenteric lymph node. These
17 data suggest that encapsulation of AF/RI may potentiate the mucosal cellular
18 immune response.

19 Figures 14 a-d show proliferative responses to AF/RI synthetic
20 peptides by rabbit Peyer's patch cells. Naive rabbits were primed twice with
21 50 micrograms of either non-encapsulated (rabbits 132 and 133) or
22 microencapsulated (rabbits 134 and 135) AF/RI pili by endoscopic
23 intraduodenal inoculation seven days apart. Seven days following the second
24 priming, Peyer's patch cells from each rabbit were cultured with AF/RI 40-55

1 (Fig.14a), AF/RI 79-94 (Fig.14b), AF/RI 108-123 (Fig.14c), or AF/RI
2 40-47/79-86 (Fig.14d) in 96-well plates for four days followed by a terminal
3 six hour pulse with [³H]thymidine. Data shown is the SI calculated from the
4 mean cpm of quadruplicate cultures. The responses of rabbits 132 and 133
5 were not significant to any of the peptides tested. Rabbit 134 had a significant
6 response to (a) AF/RI 40-55 ($p=0.0001$), (b) AF/RI 79-94 ($p=0.0280$), and
7 (d) AF/RI 40-57/79-86 ($p=0.025$), but not to (c) AF/RI 108-123. Rabbit 135
8 had a significant response to (a) AF/RI 40-55 ($p=0.034$), (b) AF/RI 79-94
9 ($p=0.040$), and (c) AF/RI 108-123 ($p<0.0001$), but not to (d) AF/RI
10 40-47/79-86. This demonstrates enhanced proliferative response to peptide
11 antigens following mucosal priming with microencapsulated pili. AF/RI pili
12 promotes RDEC-1 attachment to rabbit intestinal brush borders. Three
13 amino acid peptides were selected by theoretical criteria from the AF/RI
14 sequence as probable T or B cell epitopes and were synthesized: AF/RI 40-55
15 as a B cell epitope, 79-94 as a T cell epitope, and 108-123 as a T and B cell
16 epitope. We used these peptides to investigate a possible immunopotentiating
17 effect of encapsulating purified Af/RI pili into biodegradable, biocompatible
18 microspheres composed of polymerized lactide and glycolide at a size range
19 that promotes localization in the Peyer's Patch (5-10 micrometers). NZW
20 rabbits were primed twice with 50 micrograms AF/RI by endoscopic
21 intraduodenal inoculation and their Peyer's Patch cells were cultured in vitro
22 with the AF/RI peptides. In two rabbits which had received encapsulated
23 AF/RI, lymphocyte proliferation was observed to AF/RI 40-55 and 79-94 in
24 both rabbits and to 108-123 in one of two rabbits. No responses to any of the

1 peptides were observed in rabbits which received non-encapsulated AF/RI.
2 These data suggest that encapsulation of AF/RI may enhance the cellular
3 response to peptide antigens.

4 Figures 15a-d show B-cell responses of Peyer's patch cells to
5 AF/RI and peptides.

6 Figures 16a-d show B-cell responses of Peyer's Patch cells to
7 AF/RI and peptides.

8 Figures 17a-d show B-cell responses of spleen cells to AF/RI and
9 Peptides.

10 Figures 18a-d show B cell responses of spleen cells to AF/RI
11 and peptides.

12 Figures 15 through 18, illustrate enhanced lymphocyte antibody
13 response by mucosal immunization of rabbits with microencapsulated AF/RI
14 pilus protein. The AF/RI pilus protein has been found to be immunogenic for
15 rabbit spleen and Peyer's patch cells in vitro producing a primary IgM
16 antibody response. The purpose of this study was to determine if AR/RI pilus
17 protein immune response is enhanced by microencapsulation. The AF/RI was
18 incorporated into biodegradable, biocompatible microspheres composed of
19 lactide-glycolide copolymers, had a size range of 5-10 micrometer and
20 containing 0.62% pilus protein by weight. Initially, NZW rabbits were
21 immunized twice with 50 micrograms of either encapsulated or
22 non-encapsulated AF/RI via intraduodenal route seven days apart. For in vitro
23 challenge, 6×10^5 rabbit lymphocytes, were set in microculture at final volume
24 of 0.2 ml. Cells were challenged with AR/RI or three different synthetic 16

1 amino acid peptides representing, either predicted T, B or T and B cell
2 epitopes in a dose range of 15 to 150 ng/ml for splenic cells or 0.05 to 5.0
3 micrograms/ml for Peyer's patch mononuclear cells (in triplicate).
4 Supernatants were collected on culture days 3, 5, 7, and 9 assayed by ELISA
5 for anti-AF/R1 antibody response as compared to cell supernatant control.
6 Significant antibody responses were seen only from spleen and Peyer's patch
7 cells from rabbits immunized with microencapsulated AF/R1. The antibody
8 response tended to peak between days 5 and 9 was mainly an IgM response.
9 The results for the predicted epitopes were similar to those obtained with
10 purified AF/R1. In conclusion, intestinal immunization with AF/R1 pilus
11 protein contained within microspheres greatly enhances both the spleen and
12 Peyer's patch B-cell responses to predicted T & B-cell epitopes.

13 Figure 19 shows proliferative responses to AF/R1 40-55 by
14 rabbit MLN cells. Naive rabbits were primed twice with 50 micrograms of
15 either nonencapsulated (rabbits 132 and 133) or microencapsulated (rabbits 134
16 and 135) AF/R1 pili by endoscopic intraduodenal inoculation seven days apart.
17 Seven days following the second priming, MLN cells were cultured with
18 AF/R1 40-55 for four days in 24-well plates. Cultures were transferred into
19 96-well plates for a terminal [³H]thymidine pulse. Data shown is the SI
20 calculated from the mean cpm of quadruplicate cultures. Responses of rabbits
21 132 and 133 were not statistically significant. Responses were significant for
22 rabbits 134 ($p=0.0051$) and 135 ($p=0.0055$).

23 Figure 20 shows proliferative responses to AF/R1 40-55 by
24 rabbit spleen cells. Naive rabbits were primed twice with 50 micrograms of

1 either nonencapsulated (rabbits 132 and 133) or microencapsulated (rabbits 134
2 and 135) AF/R1 pili by endoscopic intraduodenal inoculation seven days apart.
3 Seven days following the second priming, spleen cells were cultured with
4 AF/R1 40-55 for four days in 24-well plates. Cultures were transferred into
5 96 well plates for a terminal [^3H]thymidine pulse. Data shown is the SI
6 calculated from the mean cpm of quadruplicate cultures. Responses of rabbits
7 132 and 133 were not statistically significant. Responses were significant for
8 rabbits 134 ($p=0.0.0005$) and 135 ($p=0.0066$).

9 Figure 24. A. SDS-PAGE of intact CFA/I (lane 1), trypsin
10 treated CFA/I (lane 2), and *S. aureus* V8 protease treated CFA/I. Molecular
11 masses of individual bands were estimated from molecular weight standards
12 (on left). Multiple lanes of both trypsin and V8 treated CFA/I were
13 transferred to PVDF membranes where bands corresponding to the
14 approximate molecular masses of 3500 (trypsin digest, see arrow lane 2) and
15 6000 (V8 digest, see arrow lane 3) were excised and subjected to Edman
16 degradation.²⁴ B. Resulting sequence of protein fragments from each lane of A
17 (position of sequenced portion of fragment in the intact protein. Underlined,
18 italicized residues are amino acids under dispute in literature.

19 Figure 25. ELISA assay results testing hyperimmune sera of
20 monkey²⁵(A) 2Z2 (monkey 3),²⁵(B) 184(D) (monkey 1) and²⁵(C) 34 (monkey 2)
21 to CFA/I primary structure immobilized on polyethylene pins. Monkey sera
22 diluted 1:1000. Peptide number refers first amino acid in sequence of
23 octapeptide on pin from CFA/I primary structure OD 405 refers to optical
24 density wavelength at which ELISA plates were read (405 nm).

1 Figure 26 Complete sequence of CFA/I (147 amino acids) with
2 B cell recognition site (boxed areas) as defined by each individual monkey
3 response (2Z2, 184D, and 34). Derived from data in Figure 25.

4 Figures 27-29 Lymphocyte proliferation to synthetic
5 decapeptides of CFA/I. Each monkey was immunized with three i.m.
6 injections of CFA/I subunits in adjuvant, and its spleen cells were cultured
7 with synthetic decapeptides which had been constructed using the Pepscan
8 technique. The decapeptides represented the entire CFA/I protein.
9 Concentrations of synthetic peptide used included 6.0, 0.6, and 0.06
10 micrograms/ml. Values shown represent the maximum proliferative response
11 produced by any of the three concentrations of antigen used \pm the standard
12 deviation. The cpm of the control peptide for each of the three monkeys was
13 1,518 \pm 50, 931 \pm 28, and 1,553 \pm 33 respectively. The cpm of the media
14 control for each of the three monkeys was 1,319 \pm 60, 325 \pm 13, and 1,951
15 \pm 245 respectively.

16 Figures 30-32 Lymphocyte proliferation to 6.0, 0.6, and 0.06
17 micrograms/ml synthetic decapeptides of CFA/I in one monkey. The monkey
18 (2Z2) as immunized with three i.m. injections of CFA/I subunits in adjuvant,
19 and its spleen cells were cultured with synthetic decapeptides which had been
20 constructed using the Pepscan technique. The decapeptides represented the
21 entire CFA/I protein. Values shown represent the proliferative response which
22 occurred to 6.0 micrograms/ml (Fig. 30), 0.6 micrograms/ml (Fig. 31), or
23 0.06 micrograms/ml (Fig. 32) of antigen \pm the standard deviation. The cpm of

the control peptide was $1,553 \pm 33$ and the cpm of the media control was $1,951 \pm 245$.

Figure 33 shows that rabbits numbers 21 and 22 received intraduodual administration of AF/R1 microspheres at doses of AF/R1 of 200 micrograms (ug) on day 0 and 100 ug on day 7, 14, and 21 then sacrificed on day 31. The spleen, Peyer's patch and ileal lamina propria cells at 6×10^5 in 0.2 ml in quadriplate were challenged with AF/R1 and AF/R1 1-13, 40-55, 79-94, 108-123, and 40-47, 79-85 synthetic peptides at 15, 1.5 and .15 ug/ml for 4 days. The supernatants were tested for IL-4 using the IL-4/IL-2 dependent cell line cells CT4R at 50,000/well with 0.1 ml of 6.25% supernatant for 3 days then pulsed with tritiated thymidine for 4 hrs, cells harvested and the tritiated thymidine incorporation determined, averaged and expressed with one standard deviation thousand counts per minute (kcpm).

Figure 34 shows that RDEC-1 colonization (log CFU/gm) in cecal fluids was similar in both groups (mean 6.3 vs 7.3; $p=.09$).

Figure 35 shows that rabbits given AF/R1-MS remained well and 4/6 gained weight after challenge, whereas 9/9 unvaccinated rabbits lost weight after challenge (mean weight change +10 vs -270 grams $p<.001$).

Figure 36 shows that the mean score of RDEC-1 attachment to the cecal epithelium was zero in vaccinated, and 2+ in unvaccinated animals.

Figure 37. Particle size distribution of CFA/II microsphere vaccine Lot L74F2 values are percent frequency of number or volume verses distribution. Particle size (diameter) in microns. 63% by volume are between 5-10 um and 88% by volume are less then 10 um.

1 Figure 38. Scanning electron photomicrograph of CFA/II
2 microsphere vaccine Lot L7472 standard bar represents 5 μ m distance.

3 Figure 39. Twenty-two hour CFA/II release study of CFA/II
4 microsphere vaccine Lot L7472. Percent cumulative release of CFA/II from
✓ 5 three sample: A, 33.12 mgm; B, 29.50 mgm; C, 24.20 mgm at 1, 3, 6, 8, 12
6 and 22 hour intervals. Average represents the mean \pm ISD.

7 Figure 40. Serum IgG antibody response to CFA/II microsphere
8 vaccine Lot L7472 following 2 25 μ g protein IM immunization on
9 day 0 in 2 rabbits. Antibody determined on serial dilution of sera by ELISA
10 and expressed as mean titer versus day 0, 7 and 14.

11 Figure 41. Serum IgG antibody response to CFA/II
12 microsphere vaccine Lot L7F2 following 2 25 μ g protein IM
13 immunizations on day 0 in rabbit 107 & 109. Antibody determined on serial
14 dilution (in duplicate) of sera by ELISA and expressed as mean titer versus day
15 0, 7 and 14.

16 Figure 42. Lymphocyte proliferative responses for Peyer's
17 patch cells of rabbits 65 (figure 42 (a)), 66 (figure 42 (b)), 83 (figure 42 (c)),
18 86 (figure 42 (d)), and 87 (figure 42 (e)) immunized intraduodenally with 50
19 mgm protein of CFA/II microsphere vaccine 4 and 7 days earlier. The cells
20 are challenged in vitro with CFA/II or BSA at 500, 50 and 5 μ g/ml or media
21 in triplicate. The uptake of tritiated thymidine in Kcp is expressed as mean \pm
22 ISD. Using the paired student t-test, the p values of 500 μ g/ml dose of
23 CFA/II compared to media control are: 65, p = 0.0002; 66, p = 0.0002; 83, p
24 = 0.0002; and 86, p = 0.0002.

Figure 43. Lymphocyte proliferative responses from Peyer's patch cells of rabbits 77 (figure 43(a)), 78 (figure 43(b)), 80 (figure 43(c)), 88 (figure 43(d)), and 91 (figure 43(e)) immunized intraduodenally with 50 mgm protein of CFA/II microspheres vaccine 14 and 7 days earlier. The cells are challenged in vitro with CFA with CFA/II or BSA at 500, 50 and 5 ug/ml or media in triplicate the uptake of tritiated thymidine in Kcp is expressed as mean \pm ISD. Using the paired student t -test, the protein of 500 ug/ml dose of CFA/II compared to media control are: 77, p = 0.0001; 78, p = 0.0015; 80, p = insignificant; 88, p = 0.0093; and 91 p = 0.0001.

Figure 44. ELISPOT assay of spleen cells from rabbits 65 (figure 44(a)), 66 (figure 44(b)), 83 (figure 44(c)), 86 (figure 44(d)), and 87 (figure 44(e)) immunized intraduodenally with 50 mgm protein of CFA/II microsphere vaccine 14 and 7 days earlier. These were cells placed into microculture and tested on day 0, 1, 2, 3, 4 and 5 by ELISPOT for cells secreting antibodies specific for CFA/II antigen. The results are expressed as number per 9×10^6 spleen cells versus culture day tested.

Figure 45. ELISPOT assay of spleen cells from normal control rabbits, 67, 69, 72 and 89. The cells were placed into microculture and tested on days 0, 1, 2, 3, 4 and 5 by ELISPOT for cells secreting antibodies specific for CFA/II antigen. The results are expressed as number per 9×10^6 spleen cells versus culture day tested.

Figure 46. Curve for determining vaccination dosages for regimen b.

Figure 47. Hepatitis B surface antigen release from 50:50 poly (DL-lactide-co-glycolide).

Figures 19 and 20 serve to illustrate that inclusion of Escherichia coli pilus antigen in microspheres enhances cellular immunogenicity.

FIG.48 shows a comparison of drug release from a conventional system versus a controlled release system. Peak and valley levels from conventional administrations are shown, in contrast to the steady therapeutic levels from the controlled release administration.

FIG.49 shows a scanning electron micrograph of PLGA microspheres prepared by the process described in the invention using 50/50 uncapped polymer of Mw 8-12k dalton and shows superior sphere morphology, sphere integrity, and narrow size distribution.

FIG.49 a shows a scanning electron micrograph of PLGA microspheres prepared by conventional solvent evaporation method using a 50/50 uncapped polymer of Mw 8-12k dalton.

FIG.50 shows cumulative Histatin release from PLGA microspheres, wherein release profiles from several batches are prepared using 50/50, uncapped polymer (of Mw 8-12k dalton) and wherein the process parameters are varied to modulate release between 1 and 100 days.

FIG.51 shows a scanning electron micrograph of solid, smooth

spherical surfaces of PLGA microspheres prepared by the method of in the invention using 50/50, end-capped polymer (of Mw 30-40k dalton).

FIG. 52 shows cumulative Histatin release from PLGA microspheres, wherein the release profiles are from several batches prepared using 50/50, uncapped and end-capped polymer of Mw 30-40k daltons, and wherein the process parameters are varied to modulate release between 28 to 60 days.

FIG. 53 shows cumulative Histatin release from PLGA microspheres, wherein combined release profiles from several batches have been prepared using 50/50, uncapped and end-capped polymer of Mw 8-40k daltons, while varying the process parameters to modulate release between 1 and 60 days.

FIG. 54 shows a cumulative percent release of LHRH from PLGA microspheres prepared using uncapped polymer of Mw 8-12 daltons.

1 VII. DETAILED DESCRIPTION OF THE INVENTION

2 This invention relates to the encapsulation of active core materials,
3 especially those which are medically beneficial to the mammalian animal
4 kingdom, such as biologically active agent(s), drug(s), or substance(s) within a
5 biodegradable-biocompatible polymeric matrix.

6 More precisely, applicants have discovered a medicinally beneficial
7 composition and process with the following itemized features:

- 8 1. A composition for the burst-free, sustained, programmable release of active
9 material(s) over a period from 1-100 days, which comprises: (1) An active
10 material and (2) A carrier which may contain pharmaceutically-acceptable
11 adjuvant, comprised of a blend of uncapped and end-capped biodegradable-
12 biocompatible copolymer.
- 13 2. The composition of Item 1 wherein the polymeric substance is
14 poly(lactide/glycolide).
- 15 3. The composition of Item 2, wherein the poly(lactide/glycolide) is a blend
16 of uncapped and end-capped forms, in ratios ranging from 100/0 to 1/99.
- 17 4. The composition of Item 3 wherein the copolymer (lactide to glycolide
18 L/G) ratio for uncapped and end-capped polymer is 90/10 to 40/60.
- 19 5. The composition of Item 4 wherein the copolymer (lactide to glycolide
20 L/G) ratio for uncapped and end-capped polymer is 48/52 to 52/48.
- 21 6. The composition of Item 2 wherein the molecular weight of the copolymer
22 is between 2,000-60,000 daltons.
- 23 7. The composition of Item 3 wherein the active material is biologically active
24 agent.
- 25 8. The composition of Item 7 wherein the agent is selected from the group
26 consisting essentially of antibacterial agents; peptides; polypeptides;
27 antibacterial peptides; antimycobacterial agents; antimycotic agents; antiviral

agents; hormone peptides; cardiovascular agents; narcotic antagonists;
analgesics; anesthetics; insulins; steroids including HIV therapeutic drugs
(including protease inhibitors) and AZT; estrogens; progestins; gastrointestinal
therapeutic agents; non-steroidal anti-inflammatory agents; parasympathoimetic
agents; psychotherapeutic agents; tranquilizers; decongestants; sedative-
hypnotics; non-estrogenic and non-progestional steroids; sympathomimetic
agents; vaccines; vitamins; nutrients; anti-migraine drugs; electrolyte
replacements; ergot alkaloids; anti-inflammatory agents; prostaglandins;
cytotoxic drugs; antigens; antibodies; enzymes; growth factors;
immunomodulators; pheromones; prodrugs; psychotropic drugs; nicotine;
antiblood clotting drugs; appetite suppressants/stimulants and combinations
thereof; contraceptive agents include estrogens such as diethyl silbestrol; 17-
beta-estradiol; estrone; ethinyl estradiol; mestranol; progestins such as
norethindrone; norgestryl; ethynodiol diacetate; lynestrenol;
medroxyprogesterone acetate; dimethisterone; megestrol acetate;
chlormadinone acetate; norgestimate; norethisterone; ethisterone; melentate;
norgestimate; norethisterone; ethisterone; melengestrol; norethynodrel; and
spermicidal compounds such as nonyphenoxypolyoxyethylene glycol;
benzethonium chloride; chlorindanol; include gastrointestinal therapeutic agents
such as aluminum hydroxide; calcium carbonate; magnesium carbonate;
sodium carbonate and the like; non-steroidal antifertility agents;
parasympathomimetic agents; psychotherapeutic agents; major tranquilizers
such as chloropromazine HCL; clozapine; mesoridazine; metiapine;
reserpine; thioridazine; minor tranquilizers such as chlordiazepoxide;
diazepam; meprobamate; temazepam and the like; rhinological decongestants;
sedative-hypnotics such as codeine; phenobarbital; sodium pentobarbital;
sodium secobarbital; other steroids such as testosterone and testosterone

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1 nutrient such as the essential amino acids; essential fatty acids; anti-HIV agents;
2 including AZT; antimalarials such as 4-aminoquinolines; 8 aminoquinolines;
3 pyrimethamine; anti-migraine agents such as mazindol; phentermine; anti-
4 Parkinson agents such as L-dopa; antispasmodics such as atropine;
5 methscopolamine bromide; antispasmodics and anticholinergic agents such as
6 bile therapy; digestants; enzymes and the like; antitussives such as
7 dextromethorphan and noscapine; bronchodilators; cardiovascular agents such
8 as anti-hypertensive compounds; Rauwolfia alkaloids; coronary vasodilators;
9 nitroglycerin; organic nitrites; pentaerythritetranitrate; electrolyte
10 replacements such as potassium chloride; ergotalkaloids such as ergotamine
11 with and without caffeine; hydrogenated ergot alkaloids; dihydroergocristine
12 methanesulfate; dihydroergocornine methanesulfonate; dihydroergokryptine
13 methanesulfate and combinations thereof; alkaloids such as atropine sulfate;
14 Belladonna; hyoscine hydrobromide; analgesics; narcotics such as codeine;
15 dihydrocodienone; meperidine; morphine; non-narcotics such as salicylates;
16 aspirin; acetaminophen; and d-propoxyphene; antibiotics such as the
17 cephalosporins including cefaclor and cefuroxime; chloramphenicol; gentamicin;
18 Kanamycin A. Kanamycin B; the penicillins; ampicillin; amoxicillin;
19 streptomycin A; antimycin A; chloropamtheniol; metronidazole;
20 oxytetracycline penicillin G; the tetracyclines; including minocycline; fluoro-
21 quinolones including ciprofloxacin; ofloxacin; macrolides including
22 clarithromycin; erythromycin; aminoglycosides including gentamicin;
23 amikacin; tobramycin and kanamycin; beta-lactams including ampicillin;
24 polymyxin-B; amphotericin-B; aztreonam; chloramphenicol; fusidans;
25 lincosamides; metronidazole; nitro-furantoin; imipenem/cilastatin; quinolones;
26 systemic antibiotics including rifampin; polymyxins; sulfonamides; trimethoprim;
27 glycopeptides including vancomycin; teicoplanin and imidazoles; anti-cancer

Drug 1

1 phenobarbital; trimethadione; anti-emetics such as triethylperazine;
2 antihistamines such as chlorophenazine; dimenhydrinate; diphenhydramine;
3 perphenazine; tripeleminamine and the like; anti-inflammatory agents such as
4 hormonal agents; hydrocortisone; prednisolone; prednisone; non-hormonal
5 agents; allopurinol; for claims water-soluble hormone drugs; antibiotics;
6 antitumor agents; anti inflammatory agents; antipyretics; analgesics;
7 antitussives; expectorants; sedatives; muscle relaxants; antiepileptics; anticulcer
8 agents; antidepressants; antiallergic drugs; cardiotonics; antiarrhythmic drugs;
9 vasodilators; antihypertensives; diuretics; anticoagulants; and antinarcotics; in
0 the molecular weight range of 100-100,000 daltons; indomethacin;
1 phenylbutazone; prostaglandins; cytotoxic drugs such as thiopeta; chloramucil;
2 cyclophosphamide; melphalan; nitrogen mustard; methotrexate; antigens such as
3 proteins; glycoproteins; synthetic peptides; carbohydrates; synthetic
4 polysaccharides; lipids; glycolipids; lipopolysaccharides(LPS); synthetic
5 lipopolysaccharides and with or without attached adjuvants such as synthetic
6 muramyl dipeptide derivatives; antigens of such microorganisms as *Neisseria*
7 *gonorrhoea*; *Mycobacterium tuberculosis*; *Pneumocystis carinii*; Herpes virus
8 (human types 1 and 2); Herpes zoster; *Candida albicans*; *Candida tropicalis*;
9 *Trichomonas vaginalis*; *Haemophilus vaginalis*; Group B streptococcus *ecoli*;
0 *Mycoplasma hominis*; *Haemophilus ducreyi*; *Granuloma inguinale*;
1 *Lymphogranuloma venereum*; *Treponema pallidum*; *Brucella abortus* *Brucella melitensis*
2 *Brucella suis*; *Brucella canis* *Campylobacter fetus*; *Campylobacter fetus*
3 *intestinalis*; *Leptospira pomona*; *Listeria monocytogenes*; *Brucella ovis*; Equine
4 herpes virus 1; Equine arteritis virus; IBR-IBP virus; *Chlamydia psittaci*;
5 *Trichomonas foetus*; *Toxoplasma gondii*; *Escherichia coli*; *Actinobacillus*
6 *equi*; *Salmonella abortus ovis*. *Salmonella abortus equi*; *Pseudomonas*
7 *aeruginosa*; *Corynebacterium equi*; *Corynebacterium pyogenes*; *Actinobacillus*

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Trypanosoma fipendium; Babesia cabali; Clostridium tetani; antibodies which counteract the above microorganisms; and enzymes including ribonuclease; neuramidinase; trypsin; glycogen phosphorylase; sperm lactic dehydrogenase; sperm hyaluronidase; adenosinetriphosphatase; alkaline phosphatase; alkaline phosphatase; amino peptides; trypsin chymotrypsin amylase; muramidase; acrosomal proteinase; diesterase; glutamic acid dehydrogenase; succinic and dehydrogenase; beta-glycophosphatase lipase; ATP-ase alpha-peptate gamma-glutamylotranspeptidase; steroid-beta-ol-dehydrogenase; DPN-di-aprorase; and combinations thereof.

9. The composition of Item 8 wherein the agent is selected from the group consisting essentially of antibacterial agents; antibacterial peptides; antimycobacterial agents; antimycotic agents; antiviral agents; antiparasitic agents; antifungal; hormonal peptides; cardiovascular agents; narcotic antagonist; analgesics; anesthetics; vaccines; insulins; HIV therapeutic drugs (protease inhibitors); estrogens; progestins; gastrointestinal therapeutic agents; non-steroidal anti-inflammatory agents; parasympathoimetic agents; psychotherapeutic agents; tranquilizers; decongestants; sedative-hypnotics; non-estrogenic and non-progestional steroids; sympathomimetic agents; vaccines; vitamins; nutrients; anti-malarial compounds; anti-migraine drugs; electrolyte replacements; ergot alkaloids; analgetics; non-narcotics; anti-cancer agents; anticonvulsants; anti-emetics; antihistamines; anti-inflammatory agents; prostaglandins; cytotoxic drugs; antigens; antibodies; enzymes; growth factors; immunomodulators; pheromones; prodrugs; psychotropic drugs; appetite suppressants/stimulants; and combinations thereof.

10. The composition of Item 8 wherein the agent is a peptide or polypeptide.

11. The composition of Item 10 wherein the agent is a poly peptide.

12. The composition of Item 11 wherein the molecular weight of the

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13. The composition of Item 12 wherein the polypeptide is histatin consisting of 12 amino acids and having a molecular weight of 1563.

14. The composition of Item 1 characterized by the capacity to completely release histatin in an aqueous physiological environment within from 1 to 40 days with a 100/0 blend of uncapped and end-capped poly(lactide/glycolide) having a L/G ratio of 48/52 to 52/48, and a molecular weight less than 15,000.

15. The composition of Item 14 wherein the histatin can be completely released within 18 to 40 days and the molecular weight of the poly(lactide/glycolide) is within the range of 28,000 to 40,000.

16. The composition of Item 2 characterized by the capacity to release up to 90% of the histatin in an aqueous physiological environment from 28-70 days with a 1/99 blend of uncapped and end-capped poly(lactide/glycolide) having a L/G ratio of 48/52 to 52/48 and a molecular weight range of 10,000-40,000 daltons.

17. The composition of Item 2 characterized by the capacity to release up to 80% of histatin in an aqueous physiological environment from 56-100 days with a 1/99 blend of uncapped and end-capped poly(lactide/glycolide) having a L/G ratio of 75/25 and a molecular weight of less than 15,000 daltons.

18. The composition of Item 13 having analogs of histatin with chain lengths of from 11-24 amino acids of molecular weights from 1,500-3,000 daltons and characterized by the following structures:

1. D S H A K R H H G Y K R K F H E K H H S H R G Y

2. K R H H G Y K R K F H E K H H S H R G Y R

3. K R H H G Y K R K F H E K H H S R

4. R K F H E K H H S H R G Y R

5. A K R H H G Y K R K F H

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7. K R H H G Y K R K F

*D-amino acid

19. The composition of Item 10 wherein the biologically active agent is a polypeptide Leutinizing hormone releasing hormone (LHRH) that is a decapeptide of molecular weight 1182 in its acetate form, and having the structure:

p- E H W S Y G L R P G

20. The composition of Item 13 having a molecular weight of from 1,000 to 250,000 daltons.

21. The composition of Item 2 wherein release profiles of variable rates and durations are achieved by blending uncapped and capped microspheres as a cocktail in variable amounts.

22. The composition of Item 2 wherein release of profiles of variable rates and duration are achieved by blending uncapped and capped polymer in different ratios within the same microspheres.

23. The composition of Item 12 wherein the entrapped polypeptide is any of the vaccine agents against enterotoxigenic E. coli (ETEC) selected from the group consisting of CFA/I, CFA/II, CS1, CS3, CS6 and CS17, ETEC-related enterotoxins, and combinations thereof.

24. The composition of Item 23 wherein the entrapped polypeptide consists of peptide antigens of molecular weight range of about 800-5000 daltons for immunization against enterotoxigenic E. coli (ETEC).

25. The composition of Item 24 wherein the entrapped polypeptide is selected from the group consisting essentially of an antigenic synthetic peptide containing CFA/I pilus protein T-cell epitopes; B-cell epitopes, or mixtures thereof.

26. The composition of Item 24 wherein the poly(lactide/glycolide) is a blend



1 27. The composition of Item 7 wherein said agent is selected from the group
2 consisting of water-soluble hormone drugs, antibiotics, antitumor agents, anti
3 inflammatory agents, antipyretics, analgesics antitussives, expectorants,
4 sedatives, muscle relaxants, antiepileptics, antiulcer agents, antidepressants,
5 antiallergic drugs, cardiotonics, antiarrhythmic drugs, vasodilators,
6 antihypertensives, diuretics, anticoagulants, antinarcotics, in the molecular
7 weight range of 100-100,000 daltons.

8 28. The composition of Item 1 wherein said biodegradable
9 poly(lactide/glycolide) is in an oil phase, and is present in about 1-50% (w/w).

10 29. The composition of Item 28 wherein concentration of the active agent is in
11 the range of 0.1 to about 60% (w/w).

12 30. The composition of Item 29 wherein a ratio of the inner aqueous to oil
13 phases is about 1/4 to 1/40(v/v).

14 31. The composition of Item 11 wherein the entrapped polypeptide is active at
15 a low pH, such as LHRH, adrenocorticotrophic hormone, epidermal growth
16 factor, calcitonin released polypeptide is bioactive.

17 32. The composition of Item 11 when entrapped polypeptide such as histatin is
18 inactive at a low pH, a pH-stabilizing agent of inorganic salts are added to the
19 inner aqueous phase to maintain biological activity of the released peptide.

20 33. The composition of Item 11 wherein when entrapped polypeptide such as
21 histatin is inactive at a low pH, a non-ionic surfactant such as polyoxyethylene
22 sorbitan fatty acid esters (Tween 80, Tween 60 and Tween 20) and
23 polyoxyethylene - polyoxypropylene block copolymers (Pluronic) is added to
24 the inner aqueous phase to maintain biological activity of the released
25 polypeptide.

26 34. The composition of Item 32 wherein placebo spheres loaded with the pH-
27 stabilizing agents are coadministered with polypeptide-loaded spheres to

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1 activity of the released peptide in instances where the addition of pH-stabilizing
2 agents in the inner aqueous phase is undesirable for the successful
3 encapsulation of the acid pH sensitive polypeptide.

4 35. The composition of Item 33 wherein placebo spheres loaded with non-
5 ionic surfactant are coadministered with polypeptide-loaded spheres to maintain
6 biological activity of the released peptide where the addition of non-ionic
7 surfactants in the inner aqueous phase is undesirable for successful
8 encapsulation of the acid pH sensitive polypeptide.

9 36. The composition of Item 1 comprising a blend of uncapped and capped
10 polymer, wherein complete solubilization of the copolymer leaves no residual
11 polymer at the site of administration and occurs concurrently with the complete
12 release of the entrapped agent.

13 37. A process of using composition of Item 1 for human administration via
14 parenteral routes, such as intramuscular and subcutaneous.

15 38. A process of using the composition of Item 1 for human administration
16 via topical route.

17 39. A process of using the composition of Item 1 for human administration
18 via oral routes.

19 40. A process of using the composition of Item 1 for human administration
20 via nasal, transdermal, rectal, and vaginal routes.

21 41. A process of using the composition of Item 1 for human administration in
22 the form of an oral or nasal inhalant for the respiratory tract.

23 42. A process for preparing controlled release compositions characterized by
24 burst-free, sustained, programmable release of biologically active agents,
25 comprising: Dissolving biodegradable poly(lactide/glycolide), in uncapped
26 form in methylene chloride, and dissolving a biologically active agent or active
27 core in water; adding the aqueous layer to the polymer solution and

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1 w/o emulsion in solvent-saturated aqueous phase containing a oil-in-water
2 (o/w) emulsifier; adding said w/o emulsion to an external aqueous layer
3 containing oil-in-water emulsifier to form a ternary emulsion; and stirring the
4 resulting water-in-oil-in-water (w/o/w) emulsion for sufficient time to remove
5 said solvent, and rinsing hardened microcapsules with water and lyophilizing
6 said hardened microcapsules.

7 43. The process of Item 42 wherein a solvent-saturated external aqueous phase
8 is added to emulsify the inner w/o emulsion prior to addition of the external
9 aqueous layer, to provide microcapsules of narrow size distribution range
10 between 0.05-500um.

11 44. The process of Item 42 wherein a low temperature of about 0-4 degree C
12 is provided during preparation of the inner w/o emulsion, and a low
13 temperature of about 4-20 degree C is provided during preparation of the
14 w/o/w emulsion to provide a stable emulsion and high encapsulation efficiency.

15 45. A process for preparing controlled release compositions characterized by
16 burst-free, sustained compositions characterized by burst-free, sustained,
17 programmable release of biologically active agents, comprising:

18 dissolving biodegradable poly(lactide/glycolide) in end-capped form in
19 methylene chloride, and dissolving a biologically active agent or active core in
20 water; adding the aqueous layer to the polymer solution and emulsifying to
21 provide an inner water-in-oil emulsion; stabilizing the w/o emulsion in a
22 solvent-saturated aqueous phase containing a oil-in-water (o/w) emulsifier;
23 adding said w/o emulsion to an external aqueous layer containing oil-in-water
24 emulsifier to form a ternary emulsion; and stirring a resulting water-in-oil-
25 water (w/o/w) emulsion for sufficient time to remove said solvent; and rinsing
26 hardened microcapsules with water; and lyophilizing said hardened
27 microcapsules. *chris 11/14*

46. The process of Item 42 wherein a 100/0 blend of uncapped and end-capped polymer is used to provide release of the active core in a continuous and sustained manner without a lag phase.

47. The process of Item 45 wherein a solvent-saturated external aqueous phase is added to emulsify the inner w/o emulsion prior to addition of the external aqueous layer, to provide microcapsules of narrow size distribution range between 0.05-500um.

48. The process of Item 45 wherein a low temperature of about 0-4 degree C is provided during preparation of the inner w/o emulsion, and a low temperature of about 4-20 degree C is provided during preparation of the w/o/w emulsion to provide a stable emulsion and high encapsulation efficiency.

49. A method for the protection against infection of a mammal by pathogenic organisms comprising administering orally to said mammal an immunogenic amount of an immunostimulating composition consisting essentially of an antigenic synthetic peptide encapsulated within a poly(lactide/galactide) matrix.

50. The method of Item 49 wherein the poly(lactide/glycolide) is a blend of uncapped and end-capped forms, in ratios ranging from 100/0 to 1/99.

51. The method of Item 49 wherein the poly(lactide/glycolide) is a blend of uncapped and end-capped forms in ratios ranging from 90/10 to 40/60.

52. The method of Item 49 wherein the infection is a bacterial infection.

53. The method of Item 49 where the synthetic peptide contains an epitope selected from the group consisting of CFA/I pilus protein T-cell epitopes, B-cell epitopes or mixtures thereof.

54. The method of Item 49 wherein the infection is a viral infection.

55. The method of Item 49 wherein the infection is parasitic infection.

56. The method of Item 49 wherein the infection is a fungal infection.

57. The method of Item 52 wherein the bacterial infection is caused by a

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essentially of Salmonella typhi, Shigella Sonnei, Shigella Flexneri, Shigella dysenteriae, Shigella boydii, Escheria coli, Vibrio cholera, Group D-2, Group E, Group G, Group I, Group 1, Listeria, Erysipelothrix, Mycobacterium, Aerobic pathogenic Actinomycetales, Enterobacteriaceae, Vibrio, aeromonas, Plesiomonas, Helicobacter, W. succinogenes, Acineto bacter spp., Foavobacterium, Pseudomonas, Legionella, Brucella, Haemophilus, Bordetalla, Mycoplasmas, Gardnerella, Streptobacillus, Spirillum, Calymmatobacterium, Clostridium, Treponema, Borrelia, Leptospira, Anaerobic Gram-negative Bacteria including bacilli and Cocci, Anaerobic gram-Positive Nonsporeforming Bacilli and Cocci, yersinia, staphylococcus, clostridium, Enteroccus, Streptoccus, Aerococcus, Planococcus, Stomatococcus, Micrococcus, Lactoccus, Germella, Pediococcus, Leuconostoc, Bacillus, Neisseria, Branhamella, Coryne bacterium, campylobacter, Arcanobacterium haemolyticum, Rhodococcus spp., Rhodococcus, Group A-4.

58. The method in accordance with Item 49 comprising administering orally to said mammal an immunogenic amount of a pharmaceutical composition consisting essentially of an antigenic synthetic peptide in the amount of .1 to 1%.

59. A vaccine for the immunization of a mammal against infection caused by pathogenic organisms prepared from the composition of Item 1.

60. The vaccine according to Item 59 wherein the polymeric substance is poly(DL-lactide-co-glycolide).

61. The vaccine according to Item 60 wherein the relative ratio between the lactide and glycolide (L/G) component is within the range of 40/60 to 0/100.

62. The vaccine according to Item 61 wherein the relative ratio between the amount of lactide and glycolide component is within the range of 90/10 to 40/60.

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63. A vaccine according to Item 62 wherein the pathogenic organisms are bacterial.

64. A vaccine according to Item 62 wherein the pathogenic organisms are viral.

65. A vaccine according to Item 62 wherein the pathogenic organisms are fungal.

66. A vaccine according to Item 62 wherein the pathogenic organisms are parasitic.

67. The vaccine according to Item 63 wherein the antigenic synthetic peptide is selected from the group consisting essentially of Synthetic Peptides Containing CFA/I Pilus Protein T-cell Epitopes (Starting Sequence # given)

4(Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro),

8(Thr-Ala-Ser-Val-Asp-Pro-Val-Ile-Asp-Leu),

12(Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

15(Ile-Asp-Leu-Leu-Gln-Ala-Asp-Gly-Asn-Ala),

20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

26(Pro-Ser-Ala-Val-Lys-Leu-Ala-Tyr-Ser-Pro),

72(Leu-Asn-Ser-Thr-Val-Gln-Met-Pro-Ile-Ser),

78(Met-Pro-Ile-Ser-Val-Ser-Trp-Gly-Gly-Gln),

87(Gln-Val-Leu-Ser-Thr-Thr-Ala-Lys-Glu-Phe),

126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr), and

133(Gly-Asn-Tyr-Ser-Gly-Val-Val-Ser-Leu-Val), and

mixtures thereof;

Synthetic Peptides Containing CFA/I Pilus Protein B-cell (antibody) Eptiopes (Starting Sequence # given)


3(Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val),

11(Val-Asp-Pro-Val-Idle-Asp-Leu-Leu-Gln-Ala-Asp),

clv9tkl

1 32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-
2 Glu-Ser-Tyr-Arg-Val),
3 32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe),
4 38(Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),
5 66(Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser),
6 93(Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala),
7 124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr),
8 127(Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and
9 124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-
10 Ser), and mixtures thereof; and
11 Synthetic Peptides Containing CFA/I Pilus Protein T-cell and B-cell (antibody)
12 Epitopes (Starting Sequence # given)
13 3(Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Bal-Asp-Pro),
14 8(Thr-Ala-Ser-Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-
15 Ala-Asp),
16 11(Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
17 20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),
18 124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-
19 Ser), and
20 126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and
21 mixtures thereof.

22 68. The vaccine according to Item 67 wherein the bacteria is selected from the
23 group consisting essentially of Salmonella typhi, Shigella Sonnei, Shigella
24 Flexneri, Shigella dysenteriae, Shigella boydii, Escheria coli, Vibrio cholera,
25 Group D-2, Group E, Group G, Group I, Group 1, Listeria, Erysipelothrix,
26 Mycobacterium, Aerobic pathogenic Actinomycetales, Enterobacteriaceae,
27 Vibrio, aeromonas, Plesiomonas, Helicobacter, W. succinogenes, Acineto



1 Haemophilus, Bordetella, Mycoplasmas, Gardnerella, Streptobacillus,
2 Spirillum, Calymmatobacterium, Clostridium, Treponema, Borrelia,
3 Leptospira, Anaerobic Gram-negative Bacteria including bacilli and Cocci,
4 Anaerobic gram-Positive Nonsporeforming Bacilli and Cocci, yersinia,
5 staphylococcus, clostridium, Enterococcus, Streptococcus, Aerococcus,
6 Planococcus, Stomatococcus, Micrococcus, Lactococcus, Germella, Pediococcus,
7 Leuconostoc, Bacillus, Neisseria, Branhamella, Coryne bacterium,
8 campylobacter, Arcanobacterium haemolyticum, Rhodococcus spp.,
9 Rhodococcus, Group A-4.

0 69. The vaccine according to Item 67 wherein the antigenic synthetic peptide
1 is selected from the group consisting essentially of 4(Asn-Ile-Thr-Val-thr-Ala-
2 Ser-Val-Asp-Pro),
3 8(Thr-Ala-Ser-Val-Asp-Pro-Val-Ile-Asp-Leu),
4 12(Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
5 15(Ile-Asp-Leu-Leu-Gln-Ala-Asp-Gly-Asn-Ala),
6 20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),
7 26(Pro-Ser-ala-Val-Lys-Leu-Ala-Tyr-Ser-Pro),
8 72(Leu-Asn-Ser-Thr-Val-Gln-Met-Pro-Ile-Ser),
9 78(Met-Pro-Ile-Ser-Val-Ser-Trp-Gly-Gly-Gln),
10 87(Gln-Val-Leu-Ser-Thr-Thr-Ala-Lys-Glu-Phe),
11 126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr), and
12 133(Gly-Asn-Tyr-Ser-Gly-Val-Val-Ser-Leu-Val), and mixtures thereof.

23 70. The vaccine according to Item 69 wherein the antigenic synthetic peptide is
24 4(Asn-Ile-Thr-Val-Thr-Ala-ser-Val-Asp-Pro).

71. The vaccine according to Item 69 wherein the antigenic synthetic peptide is 8(Thr-ala-ser-Val-Asp-Pro-Val-Ile-asp-Leu).

72. The vaccine according to Item 69 wherein the antigenic synthetic peptide is 12(Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp).

73. The vaccine according to Item 69 wherein the antigenic synthetic peptide is 15(Ile-Asp-Leu-Leu-Gln-Ala-Asp-Gly-Asn-Ala).

74. The vaccine according to Item 69 wherein the antigenic synthetic peptide is 20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val).

75. The vaccine according to Item 69 wherein the antigenic synthetic peptide is 26(Pro-Ser-Ala-Val-Lys-Leu-Ala-tyr-Ser-Pro).

76. The vaccine according to Item 69 wherein the antigenic synthetic peptide is 72(Leu-Asn-Ser-Thr-Val-Gln-Met-Pro-Ile-Ser).

77. The vaccine according to Item 69 wherein the antigenic synthetic peptide is 78(Met-Pro-Ile-Ser-Val-Ser-Trp-Gly-Gly-Gln).

78. The vaccine according to Item 69 wherein the antigenic synthetic peptide is 87(Gln-Val-Leu-Ser-Thr-thr-Ala-Lys-Glu-Phe).

79. The vaccine according to claim 69 wherein the antigenic synthetic peptide is 126(Ala-Gly-Thr-Ala-pro-Thr-Ala-Gly-Asn-Tyr).

80. The vaccine according to Item 69 wherein the antigenic synthetic peptide is 133(Gly-Asn-Tyr-Ser-Gly-Val-Val-Ser-Leu-Val).

81. The vaccine according to Item 67 wherein the antigenic synthetic peptide is selected from the group consisting essentially of 3(Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val),

11(Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

22(Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),

32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe),

38(Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),

66(Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser),

93(Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala),

124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr),

127(Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and

124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-

Tyr-Ser), and mixtures thereof.

82. The vaccine according to Item 81 wherein the antigenic synthetic peptide is 3(Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val).

83. The vaccine according to Item 81 wherein the antigenic synthetic peptide is 11(Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp).

84. The vaccine according to Item 81 wherein the antigenic synthetic peptide is 22(Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val).

85. The vaccine according to Item 81 wherein the antigenic synthetic peptide is 32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val).

86. The vaccine according to Item 81 wherein the antigenic synthetic peptide is 32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe).

87. The vaccine according to Item 81 wherein the antigenic synthetic peptide is 38(Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val).

88. The vaccine according to Item 81 wherein the antigenic synthetic peptide is 66(Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser).

89. The vaccine according to Item 81 wherein the antigenic synthetic peptide is 93(Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala).

90. The vaccine according to Item 81 wherein the antigenic synthetic peptide is 124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr).

91. The vaccine according to Item 82 wherein the antigenic synthetic peptide is 127(Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser).

92. The vaccine according to Item 82 wherein the antigenic synthetic peptide is 124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser).

93. The vaccine according to Item 67 wherein the antigenic synthetic peptide is selected from the group consisting essentially of 3(Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Bal-Asp-Pro),

8(Thr-Ala-Ser-Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

11(Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and

126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and mixtures thereof.

94. The vaccine according to Item 93 wherein the antigenic synthetic peptide is 3(Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Bal-Asp-Pro).

95. The vaccine according to Item 93 wherein the antigenic synthetic peptide is 8(Thr-Ala-Ser-Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp).

1 96. The vaccine according to Item 93 wherein the antigenic synthetic peptide
2 is 11(Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-ala-Asp).

3 97. The vaccine according to Item 93 wherein the antigenic synthetic peptide
4 is 20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val).

5 98. The vaccine according to Item 93 wherein the antigenic synthetic peptide
6 is 124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser).

7 99. The vaccine according to Item 93 wherein the antigenic synthetic peptide
8 is 126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser).

9 100. The method of Item 54, wherein the viral infection is caused by a virus
0 selected from the group consisting essentially of hepatitis A, hepatitis B,
1 hepatitis C, Varicella-Zoster virus, Epstein-Barr virus, Rotaviruses, polio
2 virus, human immunodeficiency virus (HIV), herpes simplex virus type 1,
3 human retroviruses, herpes simplex virus type 2, Ebola virus, cytomegalo
4 viruses, Herpes Simplex viruses, Human cytomegalovirus, Varicella-Zoster
5 Virus, Epstein-Barr Virus, Poxvirus, Influenza viruses, Parainfluenza viruses,
6 Respiratory Syncytial virus, Rhinoviruses, Coronaviruses, Adenoviruses,
7 Measles virus, Mumps virus, Rubella Virus, Human Parvoviruses,
8 Arboviruses, Rabies virus, Enteroviruses, reoviruses, Viruses Causing
9 gastroenteritis Hepatitis Viruses, Filoviruses, Arenaviruses, Papillomaviruses,
0 Polyomaviruses, Human Immunodeficiency viruses, Human Retroviruses, and
1 Spongiform Encephalopathies.

2 101. The method in accordance with Item 49 comprising administering orally
3 to said mammal an immunogenic amount of a pharmaceutical composition
4 consisting essentially of an antigen in the amount of .1 to 1%.

5 102. A vaccine for the immunization of a mammal against infection by
6 pathogenic organisms consisting essentially of an antigen in the amount of 0.1
7 to 1% encapsulated within a biodegradable-biocompatible polymeric poly(DL-

1 lactide-co-glycolide) matrix wherein the polymer is end-capped or a blend of
2 uncapped and end-capped polymers.

3 103. The vaccine according to Item 100 wherein the polymer is a blend of
4 end-capped and uncapped polymers.

5 104. The vaccine according to Item 103 wherein the relative ratio between the
6 lactide and glycolide component is within the range of 90/10 to 40/60.

7 105. The vaccine according to Item 103 wherein the relative ratio between the
8 amount of lactide and glycolide component is within the range of 48/52 to
9 52/48.

10 106. The vaccine according to Item 102 wherein the antigen is a bacteria or
11 derivatives thereof.

12 107. The vaccine according to Item 103 wherein the antigen is a virus or
13 derivatives thereof.

14 108. The vaccine according to Item 103 wherein the antigens is a parasite or
15 derivative thereof.

16 109. The vaccine according to Item 103 wherein the antigen is a fungus or
17 derivative thereof.

18 110. The vaccine according to Item 106 wherein the bacteria is selected from
19 the group consisting essentially of Salmonella typhi, Shigella Sonnei, Shigella
20 Flexneri, Shigella dysenteriae, Shigella boydii, Escheria coli, Vibrio cholera,
21 Group D-2, Group E, Group G, Group I, Group 1, Listeria, Erysipelothrix,
22 Mycobacterium, Aerobic pathogenic Actinomycetales, Enterobacteriaceae,
23 Vibrio, aeromonas, Plesiomonas, Helicobacter, W. succinogenes, Acineto
24 bacter spp., Foavobacterium, Pseudomonas, Legionella, Brucella,
25 Haemophilus, Bordetalla, Mycoplasmas, Gardnerella, Streptobacillus,
26 Spirillum, Calymmatobacterium, Clostridium, Treponema, Borrelia,
27 Leptospira, Anaerobic Gram-negative Bacteria including bacilli and Cocci, *Chro 9/16/*

1 staphylococcus, stridium, Enteroccus, Streptoccus, coccus,
2 Planococcus, Stomatococcus, Micrococcus, Lactoccus, Germella, Pediococcus,
3 Leuconostoc, Bacillus, Neisseria, Branhamella, Coryne bacterium,
4 campylobacter, Arcanobacterium haemolyticum, Rhodococcus spp.
5 Rhodococcus, Group A-4.

6 111. The vaccine of Item 107 wherein the virus is selected from the group
7 consisting essentially of hepatitis A, hepatitis B, hepatitis C, Varicella-Zoster
8 virus, Epstein-Barr virus, Rotaviruses, polio virus, human immunodeficiency
9 virus (HIV), herpes simplex virus type 1, human retroviruses, herpes simplex
0 virus type 2, Ebola virus, cytomegalo viruses, Herpes Simplex viruses, Human
1 cytomegalovirus, Varicella-Zoster Virus, Epstein-Barr Virus, Poxvirus,
2 Influenza viruses, Parainfluenza viruses, Respiratory Syncytial virus,
3 Rhinoviruses, Coronaviruses, Adenoviruses, Measles virus, Mumps virus,
4 Robella Virus, Human Parvoviruses, Arboviruses, Rabies virus, Enteroviruses,
5 reoviruses, Viruses Causing gastroenteritis Hepatitis Viruses, Filoviruses,
6 Arenaaviruses, Papillomaviruses, Polyomaviruses, Human Immunodeficiency
7 viruses, Human Retroviruses, and Spongiform Encephalopathies.

8 112. An immunostimulating composition comprising encapsulating-
9 microspheres, which may contain a pharmaceutically-acceptable adjuvant,
10 wherein said microspheres having a diameter between 1 nanogram (ng) to 10
11 microns (um) are comprised of (a) a biodegradable-biocompatible poly (DL-
12 lactide-co-glycolide) as the bulk matrix, wherein the copolymer (lactide to
13 glycolide L/G) ratio for uncapped and end-capped polymer is 0/100 to 1/99
14 and (b) an immunogenic substance comprising a bacteria, virus, fungus,
15 parasite, or derivative thereof, that serves to elicit the production of antibodies
16 in animal subjects.

1 113. An immunostimulating composition according to Item 112 wherein the
2 amount of said immunogenic substance is within the range of 0.1 to 1.5%
3 based on the volume of said bulk matrix.

4 114. An immunostimulating composition according to Item 10 wherein the
5 immunogenic substance comprises Colony Factor Antigen (CFA/II), hepatitis B
6 surface antigen (HBsAg), a mixture thereof physiologically similar antigen.

7 115. An immunostimulating composition according to Item 113 wherein the
8 relative ratio between the lactide and glycolide component is within the range
9 of 48/52 to 52/48.

10 116. An immunostimulating composition according to Item 113 wherein the
11 size of more than 50% of said microspheres is between 5 to 10 um in diameter
12 by volume.

13 117. An immunostimulating composition according to Item 113 wherein the
14 immunogenic substance is the synthetic peptide representing the peptide
15 fragment beginning with the amino acid residue 63 through 78 of Pilus Protein
16 CS3, said residue having the amino acid sequence, 63(Ser-Lys-Asn-Gly-Thr-
17 Val-Thr-Try-Ala-His-Glu-Thr-Asn-Asn-Ser-Ala).

18 118. A vaccine comprising an immunostimulating composition of Item 113
19 and a sterile, pharmaceutically-acceptable carrier therefor.

20 119. A vaccine comprising an immunostimulating composition of Item 118
21 wherein said immunogenic substance is Colony Factor Antigen (CFA/II).

22 120. A vaccine comprising an immunostimulating composition of Item 119
23 wherein said immunogenic substance is hepatitis B surface antigen (HBsAg).

24 121. A method for the vaccination against bacterial infection comprising
25 administering to a human, an antibactericidally effective amount of a
26 composition of Item 118.

27 122. A method according to Item 121 wherein the bacterial infection is caused

typhi, Shigella sonnei, Shigella Flexneri, Shigella dysenteriae, Shigella boydii,
Escheria coli, Vibrio cholera, Group D-2, Group E, Group G, Group I, Group
1, Listeria, Erysipelothrix, Mycobacterium, Aerobic pathogenic
Actinomycetales, Enterobacteriaceae, Vibrio, aeromonas, Plesiomonas,
Helicobacter, W. succinogenes, Acinetobacter spp., Foavobacterium,
Pseudomonas, Legionella, Brucella, Haemophilus, Bordetella, Mycoplasmas,
Gardnerella, Streptobacillus, Spirillum, Calymmatobacterium, Clostridium,
Treponema, Borrelia, Leptospira, Anaerobic Gram-negative Bacteria including
bacilli and Cocci, Anaerobic gram-Positive Nonsporeforming Bacilli and
Cocci, Yersinia, Staphylococcus, Clostridium, Enterococcus, Streptococcus,
Aerococcus, Planococcus, Stomatococcus, Micrococcus, Lactococcus, Germella,
Pediococcus, Leuconostoc, Bacillus, Neisseria, Branhamella, Coryne
bacterium, Campylobacter, Arcanobacterium haemolyticum, Rhodococcus spp.
Rhodococcus, Group A-4.

123. A method for the vaccination against viral infection comprising
administering to a human an antivirally effective amount of a composition of
Item 108.

124. A diagnostic assay for bacterial infections comprising a composition of
Item 7.

125. A method of preparing an immunotherapeutic agent against infections
caused by a bacteria comprising the steps of (1) immunizing a plasma donor
with a vaccine according to Item 52 such that a hyperimmune globulin is
produced which contains antibodies directed against the bacteria; (2) separating
the hyperimmune globulin and (3) purifying the hyperimmune globulin.

126. A method preparing an immunotherapeutic agent against infections
caused by a virus comprising the step of immunizing a plasma donor with a
vaccine according to Item 126 such that hyperimmune globulin is produced

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1 127. An immunotherapy method comprising the step of administering to a
2 subject an immunostimulatory amount of hyperimmune globulin prepared
3 according to Item 125.

4 128. An immunotherapy method comprising the step of administering to a
5 subject an immunostimulatory amount of hyperimmune globulin prepared
6 according to Item 125.

7 129. A method for the protection against infection of a subject by
8 enteropathogenic organisms or hepatitis B virus comprising administering to
9 said subject an immunogenic amount of an immunostimulating composition of
10 Item 112.

11 130. A method according to Item 127 wherein the immunostimulating
12 composition is administered orally.

13 131. A method according to Item 127 wherein the immunostimulating
14 composition is administered parenterally.

15 132. A method according to Item 127 wherein the immunostimulating
16 composition is administered in four separate doses on day 0, day 7, day 14,
17 and day 28.

18 133. A method according to Item 114 wherein the immunogenic substance is
19 the synthetic peptide representing the peptide fragment beginning with the
20 amino acid residue 63 through 78 of Pilus Protein CS3 said residue having the
21 amino acid sequence 63(Ser-Lys-Asn-Gly-Thr-Val-Thr-Try-ala-His-Glu-thr-asn-
22 Asn-Ser-Ala).

23 134. A method for the protection against or therapeutic treatment of bacterial
24 infection in the soft tissue or bone of a mammal comprising administering
25 locally to said mammal a bactericidally-effective amount of a composition of
26 Item 2, wherein the active material is an antibiotic which is controlled release
27 within a period of about 1 to 100 days.

1 135. The method according to Item 134 wherein the biodegradable poly(DL-
2 lactide-co-glycolide) is a blend of uncapped and end-capped forms having a
3 relative ratio between the amount of lactide and glycolide component within
4 the range of 100/0 to 1/99.

5 136. A method according to Item 135 wherein the bacterial infection is (1) a
6 subcutaneous infection secondary to contaminated abdominal surgery, (2) an
7 infection surrounding prosthetic devices and vascular grafts, (3) ocular
8 infections, (4) topical skin infections, (5) orthopedic infections, including
9 osteomyelitis, and (6) oral infections.

0 137. The method according to Item 136 wherein the oral infections are
1 pericoronitis or periodontal disease.

2 138. The method according to Item 135 wherein the administration is effected
3 prior to infection.

4 139. The method according to Item 135 wherein the administration is effected
5 subsequent to infection.

6 140. The method according to Item 135 wherein said animal is a human.

7 141. The method according to Item 135 wherein said animal is a nonhuman.

8 142. The method in accordance with Item 135 comprising applying to the soft
9 tissue or bone tissue of said animal a bactericidally-effective amount of a
10 pharmaceutical composition consisting essentially of an antibiotic in the ant,
11 selected from the group consisting of a beta-lactam, aminoglycolide,
12 polymyxin-b, Amphotericin B, Aztreonam, cephalosporins, chloramphenicol,
13 fusidans, lincosamides, macrolides, methronidazole, nitro-furation,
14 Imipenem/cilastin, quinolones, refampin, polyenes, tetracycline, sulfonamides,
15 trimethoprim, vancomycin, teicoplanin, imidazoles, and erythromycin,
16 encapsulated within a biodegradable poly(DL-lactide-co-glycolide) polymeric
17 matrix, wherein the amount of the lactide and glycolide (L/G) component is

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Ans 9 20

1 matrix which is present in the amount of from 40 to 100 percent, resulting in
2 the controlled release of a bacteriacidal amount of the said antibiotic over a
3 period of from 1 to 100 days.

4 143. The method of Item 142 wherein the polymeric matrix consists
5 essentially of a poly(DL-lactide-co-glycolide) wherein the relative ratio
6 between the amount of lactide and glycolide (L/G) component is within the
7 range of 48/52 to 52/48.

8 144. The method of Item 142 wherein the bacterial infection is caused by a
9 resistant or non-resistant bacteria selected from the group consisting essentially
10 of Enterobacteriaceae; Klebsiella sp.; Bacteroides sp. Enterococci; Proteus sp.;
11 Streptococcus sp.; Staphylococcus sp.; Pseudomonas sp.; Neisseria sp.;
12 Pedptostreptococcus sp.; Fusobacterium sp.; Actinomyces sp.; Mycobacterium -
13 sp.; Listeria sp.; Corynebacterium sp.; Propionibacterium sp.; Actinobacillus
14 sp.; Aerobacter sp.; Borrelia sp.; Campylobacter sp.; cytophaga sp.;
15 Pasteurella sp.; Clostridium sp., Enterobacter aerogenes, Peptococcus sp.,
16 Proteus vulgaris, Proteus morganii, Staphylococcus aureus, Streptococcus
17 pyogenes, Actinomyces sp., Campylobacter fetus, and Legionella
18 pneumophila, ampicillin-resistant strain of S. aureus, and methicillin-resistant
19 strain of S. aureus.

20 145. The method of Item 142 wherein the antibiotic is selected from the group
21 consisting essentially of a beta-lactam, aminoglycoside, polymyxin-B,
22 amphotericin B, aztreonam, cephalosporins, chloramphenicol, fusidans,
23 lincosamides, macrolides, methronidazole, nitro-furantoin, Imipenem/cilastin,
24 quinolones, rifampin, polyenes, tetracycline, sulfonamides, trimethoprim,
25 vancomycin, teicoplanin, imidazoles, and erythromycin.

26 146. The method of Item 145 wherein the beta-lactam is cephalosporin.

27 147. The method of Item 145 wherein the beta-lactam is penicillin.

1 149. The method of Item 145 wherein the aminoglycoside is amikacin.

2 150. The method of Item 145 wherein the aminoglycoside is tobramycin.

3 151. The method of Item 145 wherein the aminoglycoside is kanamycin.

4 152. The method of Item 145 wherein the beta-lactam is an ampicillin.

5 153. The method of Item 152 wherein the polymeric matrix consists

6 essentially of a poly(DL-lactide-co-glycolide) wherein the relative ratio

7 between the amount of lactide and glycolide (L/G) component is within the

8 range of 48/52 to 58/42.

9 154. The method of Item 152 wherein the ampicillin is present in an amount

10 of from 5 to 60 percent and the amount of polymeric matrix is from 40 to 95

11 percent.

12 155. The process of using the composition of Item 1 to treat humans in need,

13 thereof, suffering from diseases and/or ailments from the group consisting of:

14 viral infections; bacterial infections; fungal infections; parasitic infections and

15 more specific diseases and/or ailments; such as as, aids; alzheimer's dementia;

16 angiogenesis diseases; aphthous ulcers in AIDS patients; asthma; atopic

17 dermatitis; psoriasis; basal cell carcinoma; benign prostatic hypertrophy; blood

18 substitute; blood substitute in surgery patients; blood substitute in trauma

19 patients; breast cancer; breast cancer; cutaneous & metastatic; cachexia in

20 AIDS; campylobacter infection; cancer; pneumonia; sexually transmitted

21 diseases (STDs); cancer; viral diseases; candida albicans in AIDS and cancer;

22 candidiasis in HIV infection; pain in cancer; pancreatic cancer; parkinson's

23 disease; peritumoral brain edema; postoperative adhesions (prevent);

24 proliferative diseases; prostate cancer; ragweed allergy; renal disease;

25 restenosis; rheumatoid arthritis; rheumatoid arthritis; allergies; rotavirus

26 infection; scalp psoriasis; septic shock; small-cell lung cancer; solid tumors;

27 stroke; thrombosis; type I diabetes; type I diabetes w/kidney transplants; type

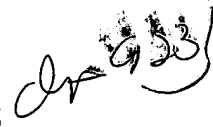
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1 rhythm disorders; central nervous system diseases; central nervous system
2 disorders; cervical dystonia (spasmodic torticollis); choroidal neovascularization;
3 chronic hepatitis c, b and a; colitis associated with antibiotics; colorectal
4 cancer; coronary artery thrombosis; cryptosporidiosis in AIDS;
5 cryptosporidium parvum diarrhea in AIDS; cystic fibrosis; cytomegalovirus
6 disease; depression; social phobias; panic disorder; diabetic complications;
7 diabetic eye disease; diarrhea associated with antibiotics; erectile dysfunction;
8 genital herpes; graft-vs host disease in transplant patients; growth hormone
9 deficiency; head and neck cancer; head trauma; stroke; heparin neutralization
10 after cardiac bypass; hepatocellular carcinoma; HIV; HIV infection;
11 huntington's disease; CNS diseases; hypercholesterolemia; hypertension;
12 inflammation; inflammation and angiogenesis; inflammation in cardiopulmonary
13 bypass; influenza; migraine head ache; interstitial cystitis; kaposi's sarcoma;
14 kaposi's sarcoma in AIDS; lung cancer; melanoma; molluscum contagiosum in
15 AIDS; multiple sclerosis; neoplastic meningitis from solid tumors; non-small
16 cell lung cancer; organ transplant rejection; osteoarthritis; rheumatoid arthritis;
17 osteoporosis; drug addiction; shock; ovarian cancer; Amebiasis; Babesiasis;
18 Chagas' disease (Trypanosoma cruzi); Cryptosporidiosis; Cysticercosis;
19 Fascioliasis; Filariasis; Echinococcosis; Giardiasis; Leishmaniasis; Malaria;
20 Paragonimiasis; Pneumocystosis; Schistosomiasis; Strongyloidiasis;
21 Toxocariasis; Toxoplasmosis; Trichinellosis; Trichomoniasis; yeast infection;
22 and pain.

23 156. A vaccine for prepared from the composition of Item 1 to prevent the
24 occurrence in humans of diseases and/or ailments comprising viral infections;
25 bacterial infections; fungal infections; parastic infections and more specific
26 diseases and/or ailments; such as as, aids; alzheimer's dementia; angiogenesis
27 diseases; aphthour ulcers in AIDS patients; asthma; atopic dermatitis;

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1 blood substitute in surgery patients; blood substitute in trauma patients; breast
2 cancer; breast cancer; cutaneous & metastatic; cachexia in AIDS;
3 campylobacter infection; cancer; pneumonia; sexually transmitted diseases
4 (STDs); cancer; viral diseases; candida albicans in AIDS and cancer;
5 candidiasis in HIV infection; pain in cancer; pancreatic cancer; parkinson's
6 disease; peritumoral brain edema; postoperative adhesions (prevent);
7 proliferative diseases; prostate cancer; ragweed allergy; renal disease;
8 restenosis; rheumatoid arthritis; rheumatoid arthritis; allergies; rotavirus
9 infection; scalp psoriasis; septic shock; small-cell lung cancer; solid tumors;
10 stroke; thrombosis; type I diabetes; type I diabetes w/kidney transplants; type
11 II diabetes; visceral leishmaniasis; malaria; periodontal or gum disease; cardiac
12 rhythm disorders; central nervous system diseases; central nervous system
13 disorders; cervical dystonia (spasmodic torticollis); choroidal neovascularization;
14 chronic hepatitis c, b and a; colitis associated with antibiotics; colorectal
15 cancer; coronary artery thrombosis; cryptosporidiosis in AIDS;
16 cryptosporidium parvum diarrhea in AIDS; cystic fibrosis; cytomegalovirus
17 disease; depression; social phobias; panic disorder; diabetic complications;
18 diabetic eye disease; diarrhea associated with antibiotics; erectile dysfunction;
19 genital herpes; graft-vs host disease in transplant patients; growth hormone
20 deficiency; head and neck cancer; head trauma; stroke; heparin neutralization
21 after cardiac bypass; hepatocellular carcinoma; HIV; HIV infection;
22 huntington's disease; CNS diseases; hypercholesterolemia; hypertension;
23 inflammation; inflammation and angiogenesis; inflammation in cardiopulmonary
24 bypass; influenza; migraine head ache; interstitial cystitis; kaposi's sarcoma;
25 kaposi's sarcoma in AIDS; lung cancer; melanoma; molluscum contagiosum in
26 AIDS; multiple sclerosis; neoplastic meningitis from solid tumors; non-small
27 cell lung cancer; organ transplant rejection; osteoarthritis; rheumatoid arthritis;



1 Chagas' disease (Trypanosoma cruzi); Cryptosporidiosis; Cysticercosis;
2 Fascioliasis; Filariasis; Echinococcosis; Giardiasis; Leishmaniasis; Malaria;
3 Paragonimiasis; Pneumocystosis; Schistosomiasis; Strongyloidiasis;
4 Toxocariasis; Toxoplasmosis; Trichinellosis; Trichomoniasis; yeast infection;
5 and pain.

6 As noted, in the Summary of the Invention section herein, a discussion
7 of this invention will be presented as Phases I, II and III.

8 PHASE I

Invention Presents

9 This illustrative phase of the invention presents the novel use of a
0 pharmaceutical composition, a micro- or macrocapsule/sphere formulation,
1 which comprises an antibiotic encapsulated within a biodegradable polymeric
2 matrix such as poly (DL-lactide-co-glycolide) (DL-PLG) in the effective
3 pretreatment of mammals to prevent bacterial infections and the posttreatment
4 of mammals (including humans and non-human mammals) with bacterial
5 infections. Microcapsules and microspheres are usually powders consisting of
6 spherical particles of 2 millimeter or less in diameter, usually 500 micrometer
7 or less in diameter. If the particles are less than 1 micron, they are often
8 referred to as nanocapsules or nanospheres. For the most part, the difference
9 between microcapsules and nanocapsules is their size; their internal structure
0 is about the same. Similarly, the difference between microspheres and
1 nanospheres is their size; their internal structure is about the same.

22 A microcapsule (or nanocapsule) has its encapsulated material,
23 herein after referred to as agent, centrally located within a unique membrane,
24 usually a polymeric membrane. This membrane may be termed a
25 wall-forming material, and is usually a polymeric material. Because of their
26 internal structure, permeable microcapsules designed for controlled-release
27 applications release their agent at a constant rate (zero-order rate of release).

1 Hereinafter, term microcapsule will include nanocapsules, and particles in
2 general that comprise a central core surrounded by a unique outer membrane.

3 A microsphere has its agent dispersed throughout the particle;
4 that is, the internal structure is a matrix of the agent and excipient, usually a
5 polymer excipient. Usually controlled-release microspheres release their agent
6 at a declining rate (first-order). But microspheres can be designed to release
7 agents at a near zero-order rate. Microspheres tend to be more difficult to
8 rupture as compared to microcapsules because their internal structure is
9 stronger. Hereinafter, the term microspheres will include nanospheres,
10 microparticles, nanoparticles, microsponges (porous microspheres) and
11 particles in general, with an internal structure comprising a matrix of agent
12 and excipient.

13 One can use other terms to describe larger microcapsules or
14 microspheres, that is, particles greater than 500 micrometer to 7 millimeter or
15 larger. These terms are macrocapsules, macrospheres, macrobeads and
16 beads. Macrocapsules, macrospheres, macrobeads and beads will be used
17 interchangeably herein.

18 More particularly, the applicants have discovered efficacious
19 pharmaceutical compositions wherein the relative amounts of antibiotic to the
20 polymer matrix are within the ranges of 5 to 60 preferred that relative ratio
21 between the lactide and glycolide component of the
22 poly(DL-lactide-co-glycolide) is within the range of 40:60 to 100:0, most
23 preferably. Applicants' most preferred composition consists essentially of 30
24 to 40(core loading) and 60 to 70 poly(DL-lactide-co-glycolide) (DL-PLG).
25 However, it is understood that effective core loads for other antibiotics will be
26 influenced by the nature of the drug, the microbialetiology and type of
27 infection being prevented and/or treated. From a biological perspective, the

1 minimal inflammatory response, is biologically compatible, and degrades
2 under physiologic conditions to products that are nontoxic and readily
3 metabolized. Similar polymeric compositions which afford in vitro release
4 kinetics, as discussed below for DL-PLG, are considered by applicants to be
5 within the scope of this invention. Applicants have discovered that antibiotic
6 encapsulated microcapsules/spheres or macrocapsules/spheres (beads) having a
7 diameter within the range of about 40 microns to about 7 millimeters to be
8 especially useful in the practice of this invention.

9 Surprisingly, applicants have discovered an extremely effective
10 method of treating bacterial infections of soft-tissue or (bone osteomyelitis)
11 and preventing these type infections with antibiotics such as beta-lactams,
12 aminoglycosides, polymyxin-B, amphotericin B, aztreonam, cephalosporins,
13 chloramphenicol, fusidans, lincosamides, macrolides, metronidazole,
14 nitro-furantion, Imipenem/cilastin, quinolones, rifampin, polyenes,
15 tetracycline, sulfonamides, trimethoprim, vancomycin, teicoplanin,
16 imidazoles, and erythromycin 1) micro- and macroencapsulated or 2) micro-
17 and macrospheres formulated within a polymeric matrix such as a
18 poly(DL-lactide-co-glycolide), which has been formulated to release the
19 antibiotic at a controlled, programmed rate over a desirable extended period
20 of time. The microcapsules/spheres have been found to be effective when
21 applied locally, including topically, to open contaminated wounds thereby
22 facilitating the release of the antibiotic from multiple sites within the tissue in
23 a manner which concentrates the antibiotic in the area of need. Similarly, the
24 encapsulated antibiotics of this invention both in the microcapsule/sphere and
25 macrocapsule/sphere (bead) form are effective for the prevention and
26 treatment of orthopedic infections that include osteomyelitis, contaminated
27 open fractures, and exchange revision arthroplasty. The macrocapsules/sphere

1 addition the object to the surgeon of using the subject invention as a packing
2 material for dead space. The subject invention offers an optimal treatment for
3 orthopaedic infections because release of the antibiotic from the micro- or
4 macrocapsule/sphere is completely controllable over time; antibiotic can be
5 encapsulated into the sphere; the sphere can be made of any size; and unlike
6 the methylmethacrylate beads, the subject invention biodegrades over time to
7 nontoxic products and does not have to be surgically removed from the treated
8 site. Since virtually any antibiotic can be encapsulated into the polymer the
9 instant invention can be used to sustain release all known antibiotics.

10 Applicants have discovered and/or contemplate that local
11 application of microencapsulated or macroencapsulated antibiotic provides
12 immediate, direct, and sustained dosing which targets the antibiotic to the pre-
13 or post infected soft-tissue or bone site, and minimizes problems inherent in
14 systemic drug administration. It appears to applicants that there is a
15 significant reduction of nonspecific binding of antibiotic to body proteins,
16 while in route to targeted sites when the antibiotic has been encapsulated in
17 accordance with this invention. Additionally, antibiotics with short half-lives
18 can be used more efficiently, undesirable side-effects can be minimized, and
19 multiple dosing can be eliminated. These attributes satisfy a long-felt need to
20 improve the effectiveness and predictability of drug delivery to accomplish the
21 desired clinical result in patients.

22 The ability to concentrate the antibiotic within the wound site
23 ensures an extended period of direct contact between an effective antibiotic
24 level and the infecting microorganisms. Many drugs have a therapeutic range
25 below which they are ineffective and above which they are toxic. Oscillating
26 drug levels, commonly observed following systemic administration, may cause
27 alternating periods of ineffectiveness and toxicity. A single dose of

1 desired therapeutic range. Applicants have discovered that microencapsulated
2 or macroencapsulated heavy concentrated doses of antibiotics are effective for
3 the treatment and prevention of infections caused by antibiotic-resistant
4 bacteria.

5 Topical application of the antibiotic microcapsule/ sphere
6 formulation to infected wounds allows local application of the antibiotic in a
7 single dose, whereby an initial burst of antibiotic for immediate soft- or
8 hard-tissue perfusion, followed by a prolonged, effective level of antibiotic is
9 achieved in the tissue at the wound site. Applicants contemplate herein
10 antibiotic microcapsules/spheres and macrocapsules/spheres consisting of an
11 antibiotic and DL-PLG and the summarized results of illustrative experiments,
12 that evaluated the prototype microcapsules in vitro and in vivo.

13 The subject invention is successful in preventing and treating
14 (1) soft-tissue infections, (2) osteomyelitis, and (3) infections surrounding
15 internally fixed fractures. These results were confirmed using the
16 microcapsule/sphere form of the encapsulated antibiotics. The
17 microcapsule/sphere and macrocapsule/sphere are also of value in numerous
18 other applications including soft-tissue infections that involve, but are not
19 limited to the prevention and treatment of (1) subcutaneous infections
20 secondary to contaminated abdominal surgery, (2) infections surrounding
21 prosthetic devices and vascular grafts, (3) ocular infections, (4) topical skin
22 infections, and (5) in oral infections such as pericoronitis and periodontal
23 disease.

24 The biodegradation rate of the excipient is controllable because
25 it is related to the mole ratio of the constituent monomers, the excipient
26 molecular weight and the surface area of the microcapsules produced.
27 Microcapsules/spheres with diameters of 250 micrometers or less are

1 aerosol sp The macrocapsules/spheres are manually placed in the tissue
2 on bone by the surgeon at the time of surgical debridement. Due to the
3 unique pharmacokinetic advantages realized with the continuous delivery of
4 antibiotic into tissue from a controlled-release vehicle, applicants have found
5 that a small total dose is required to obtain an optimal therapeutic effect.

6 VII. EXAMPLES

7 The herein offered examples provide methods for illustrating,
8 without any implied limitation, the practice of this invention in the treatment
9 of bacterial wound infections.

0 The profile of the representative experiments have been chosen
1 to illustrate the antibacterial activity of antibiotic-polymeric matrix
2 composites.

3 All temperatures not otherwise indicated are in degrees Celcius
4 (°C) and parts or percentages are given by weight.
5

MATERIALS AND METHODS

A. Microcapsules/spheres. The ampicillin anhydrate microspheres used in these studies (Composite Batch D 856-038-1) consisted of 30.7 wt in a copolymer of 52:48 poly (DL-lactide-co-glycolide). The size of the microspheres ranged from 45 to 150 microns and they were sterilized with 2.0 Mrad of gamma irradiation.

Animals. New Zealand white rabbits (Dutchland Laboratories, Denver, Pa.), weighing 2.0 to 2.5 kg each, were used. The animals were housed in individual cages and were fed a standard laboratory diet. The experiments described herein were conducted in accordance with the principles set forth in the Guide for the Care and Use of Laboratory Animals.

EXAMPLE 1

Osteomyelitis Model. The technique used to produce osteomyelitis was a modification of the procedure described previously by Norden. Briefly, New Zealand white rabbits (2.0 - 2.5 kg, each) were anesthetized with ketamine hydrochloride and xylazine and access to the medullary canal was gained by inserting an 18-gauge Osgood needle (Becton Dickinson Corp., Rutherford, NJ) into the right proximal tibial metaphysis. Through this needle was injected 0.1 ml of 5 Pharmaceuticals, Tenaflly, NJ) followed by injection of approximately 5×10^6 CFU of S. aureus ATCC 6538P.

The hole in the bone was sealed with bone wax and each animal received a single subcutaneous injection of 3-ml TORBUTROL™ (A. J. Buck, Hunt Valley, MD) for postoperative pain control. Antibiotic therapy was then initiated either immediately or was delayed for 7-days as described in detail below.

EXAMPLE 2

Immediate Antibiotic Therapy. The initial experiment was designed to evaluate the efficacy of local therapy with microencapsulated ampicillin for the prevention of experimental osteomyelitis. A total of 31 rabbits were infected in the right proximal tibia with sodium morrhuate and S. aureus and treatment was initiated immediately as follows:

Group A (n = 6) received three daily subcutaneous injections (75 mg/kg/day) of aqueous sodium ampicillin (Polycillin-N™, Bristol Laboratories, Syracuse, NY) at 8-hour intervals for 14 consecutive days;]

Group B (n = 7) received a single intramedullary injection of 100 mg of microencapsulated ampicillin containing an equivalent of 30.7 mg of ampicillin anhydrate. The microcapsules/spheres were suspended in 0.2-ml of 2injection vehicle) and were then injected into the medullary canal through the same needle that was used to inject the sclerosing agent and bacteria;

Group C (n = 4) received a single intramedullary injection of 0.12 ml (30.7 mg) of aqueous sodium ampicillin (representing the unencapsulated free drug); and

Groups D, E, and F (n = 14) served as controls and received either an intramedullary injection of placebo microcapsules (100 mg) without antibiotic; injection vehicle (0.2 ml) without antibiotic; or no treatment.

The animals were observed for a total of 8-weeks during which time roentgenograms were obtained to evaluate the progression of the disease. All surviving animals were euthanized intravenously at two months postinfection with T-61 euthanasia solution (1 mg/kg/iv) and the tibiae were harvested for bacteriological analysis as described below.

EXAMPLE 3

Delayed Antibiotic Therapy Without Debridement. In the second experiment, a total of 30 rabbits were injected in the right proximal tibia with sodium morrhuate and S. aureus and the infection was allowed to become established for 7-days. On Day 7, the animals were reanesthetized and an incision was made over the patellar tendon to expose the tibial tuberosity. A 5-mm drill hole was made in the tibial tuberosity and a trocar, measuring approximately 15 centimeters in length, was inserted into the medullary canal to obtain a marrow specimen for culture. The animals were then randomly assigned to the following treatment

groups:

Group A (n = 8) received three daily subcutaneous injections of aqueous sodium ampicillin (75mg/kg/day) at 8-hour intervals for 14-days;

Group B (n = 8) received an intramedullary application of 150 mg of microencapsulated ampicillin containing an equivalent of 45 mg of ampicillin anhydrate. The microcapsules were initially suspended in 0.2 ml of the injection vehicle and then aspirated into a sterile trocar. The trocar was then inserted into the medullary canal through the drill hole in the tibial tuberosity;

Group C (n = 8) received an intramedullary application of 0.18 ml (45 mg) of aqueous sodium ampicillin which was also delivered into the canal with a trocar; and

Group D (n = 6) served as controls and received no treatment.

Following the implantation of the antibiotics into the medullary canal, the hole in the tibial tuberosity was sealed with bone wax and the incision site was closed with 3-0 Dexon sutures.

All of the surviving animals were euthanized 8 weeks following the initiation of treatment and the tibiae were harvested for

bacteriological analysis.

EXAMPLE 4

Delayed Antibiotic Therapy With Debridement. Because standard treatment of chronic osteomyelitis requires the surgical removal of devitalized osseous tissue, the objective of this experiment was to evaluate the efficacy of local antibiotic therapy with microencapsulated ampicillin anhydrate when used in conjunction with debridement. A total of 30 rabbits were injected in the right proximal tibia with sodium morrhuate and S. aureus and the infection was allowed to establish for 7 days. On Day 7 each animal underwent a standardized surgical debridement of the infected tibia. The animals were anesthetized and an incision was made to expose the medial aspect of the tibia. A Hall drill was used to decorticate approximately one-third of the bone thereby creating a channel that extended the length of the bone. The canal was thoroughly debrided with a curette and then irrigated with 20 ml of sterile saline. Cultures of the marrow were obtained at this time for bacteriological analysis. Immediately following completion of the debridement procedure, the animals were randomly assigned to the following treatment groups:

Group A (n = 10) received 150 mg of microencapsulated ampicillin containing an equivalent of 45 mg of ampicillin anhydrate. The microcapsules were suspended in 0.2-ml of injection vehicle and were then implanted into the debrided canal

with a sterile trocar;

Group B (n = 10) received 45 mg of unencapsulated sodium ampicillin in powder form which was applied uniformly into the debrided canal; and

Group C (n = 5) and Group D (n = 5) served as controls and received either an intramedullary application of placebo microcapsules (150 mg) without antibiotic or (2) an injection vehicle (0.2 ml) without antibiotic, respectively.

Immediately following the implantation of the materials into the medullary canal, the incision site was closed with 3-0 Dexon sutures and each animal received 3-ml of Torbutrol™ for 3 consecutive days for postoperative pain. The animals were euthanized at 8 weeks following the initiation of treatment and the tibiae were harvested for bacteriological evaluation.

EXAMPLE 5

Roentgenographic Evaluation. Radiographs of the infected tibiae were obtained at various time intervals and were evaluated by a board certified skeletal radiologist (LMM) using a grading system that was originally developed by Norden et al. Four radiographic parameters (sequestrum formation, periosteal reaction, bone destruction, and extent of disease) were evaluated for each animal and a numerical value was assigned for each variable. The scores were then totaled to arrive at an overall

radiographic severity score. The highest total score possible with this grading scheme was +7 and reflected the maximum degree of radiographic severity.

EXAMPLE 6

Cultures of Bone. For bacteriological evaluation, the tibiae were dissected free of adherent soft-tissue and the surface of the bone was cleaned with alcohol pads. The bone was then weighed and crushed to small pieces with a sterile mortar and pestle. The crushed bone was suspended in 5 ml of sterile saline and serial 10-fold dilutions were prepared in 0.1 Each dilution (0.1 ml) was then streaked onto both sheep blood agar and mannitol salt agar plates which were incubated aerobically at 37°C for 24 hours. The recovery of any S. aureus colonies from the bones was evidence of a persistent osseous infection and was considered as a treatment failure.

EXAMPLE 7

Measurement of Serum Ampicillin Levels. In the experiment where local antibiotic therapy was used in conjunction with debridement, serum levels of ampicillin were measured for all of the animals treated with either an intramedullary application of microencapsulated ampicillin anhydrate (Group A) or unencapsulated free drug (Group B). Serum was obtained from all animals at 1 hour, 1 day, and 7 days following the implantation of the antibiotics into the tibiae and serum ampicillin levels were

measured using the agar-well diffusion assay described previously in detail by Bennett et al. A standard curve was constructed relating the size of the zones of inhibition obtained with a series of ampicillin standards tested against Sarcina lutea ATCC 9341 as the reference organism. Ampicillin concentrations in the test sera were then calculated from this standard curve.

RESULTS OF EXAMPLES 1 THROUGH 7

Immediate Antibiotic Therapy. The results of the initial experiment showing the effect of immediate parenteral versus local ampicillin therapy for the prevention of experimental osteomyelitis are presented in Table 2. Radiographic changes were initially detected in the control animals (Groups D, E, and F) at 2 weeks postinfection and consisted predominantly of periosteal reaction. By 7 weeks, however, the majority of the control animals (75 scores ranging from +5.25 to +7.00 indicating extensive osseous involvement. Radiographic evidence of osteomyelitis was absent in animals that received either a 14 day course of parenteral ampicillin therapy (Group A) or those that received an intramedullary injection of unencapsulated ampicillin (Group C). Only a minimal periosteal reaction was noted at day 42 for Group B animals that received an intramedullary injection of microencapsulated ampicillin, however, all other radiographic parameters were found to be within normal limits. Cultures of the tibiae at 8 weeks following the initiation of treatment showed that all of the animals treated with either a 14 day course of

parenteral ampicillin therapy or a single intramedullary injection of microencapsulated ampicillin had sterile bone cultures. Free unencapsulated ampicillin, injected locally into the bone, was also effective and sterilized the tibiae of 3 of 4 (75%). In contrast, all 13 surviving control animals in Groups D, E, and F developed culture-positive osteomyelitis with S. aureus counts ranging from 1.3×10^6 to 2.0×10^7 CFU recovered per gram of bone.

Delayed Antibiotic Therapy Without Debridement. Table 3 shows the results of the experiment when antibiotic therapy was delayed for 7 days postinfection and was then initiated without debridement. Of the 8 animals in Group A that received a 14 day course of parenteral ampicillin therapy, 6 (75% aureus bone cultures). Only 2 of these animals survived the entire length of the experimental protocol; six animals died within 1-2 weeks of completing their antibiotic therapy after developing profuse diarrhea. Of the 7 surviving rabbits in Group C that received an intramedullary application of 45 mg of unencapsulated ampicillin, 5 (71% with a single intramedullary application of microencapsulated ampicillin anhydrate (Group B) sterilized the tibiae of 4 of 8 (50% of S. aureus recovered from the tibiae of the other animals in this group as compared with the controls (Group D). All of the control animals developed osteomyelitis with an average of 2.8×10^5 CFU of S. aureus recovered per gram of bone. A Chi square analysis of the proportion of animals in each treatment group with positive bone cultures showed no statistically significant

differences among the groups ($p = 0.23$).

Delayed Antibiotic Therapy With Debridement. In this experiment we evaluated the effect of local antibiotic therapy when used in conjunction with debridement for the treatment of a 7-day established osseous infection. Bacteriological cultures of the tibiae at the time of debridement (before antibiotic therapy was initiated) yielded S. aureus in 29 of 30 (97 shown in Table 4, all 10 of the animals in Group A that were treated with debridement plus microencapsulated ampicillin anhydrate had sterile bone cultures. In contrast, of the 10 animals in Group B that were treated with debridement plus unencapsulated ampicillin only 3 had sterile bone cultures whereas 7 developed culture-positive osteomyelitis. A Chi square analysis showed a statistically significant difference ($p < 0.01$) in the proportion of animals with sterile bone cultures in the microencapsulated ampicillin treated group as compared with the group that was treated with the unencapsulated form of the antibiotic. Debridement alone, without local antibiotic therapy, was not effective for the treatment of this established osseous infection with all 10 control animals (Groups C and D) developing culturepositive osteomyelitis.

Serum Ampicillin Levels. In the experiment where local antibiotic therapy was initiated in conjunction with debridement, serum concentrations of ampicillin were measured for all animals

that received either an intramedullary application of microencapsulated ampicillin anhydrate or an equivalent dose of unencapsulated free ampicillin. The data is presented in Figure 1. Serum levels of ampicillin were only detected at 1-hour after the implantation of the antibiotics into the tibiae. At this time interval, the mean serum concentration of ampicillin in the Group B animals that received 45 mg of unencapsulated ampicillin (0.79 ± 0.24 micrograms/ml) was approximately 7-fold higher than the mean serum ampicillin concentration of the Group A animals that received an equivalent dose of the microencapsulated form of the antibiotic (0.11 ± 0.08 micrograms/ml).

DISCUSSION RELATED TO EXAMPLES 1 THROUGH 7

Previous attempts to develop a biodegradable antibiotic delivery system for the local treatment of bone infections have met with only limited success. Zilch and Lambiris reported on the treatment of 46 patients with chronic osteomyelitis using a biodegradable fibrin-cefotaxim compound that was implanted into the bone at the time of surgical intervention and reported healing in only 67. A limitation of this system was the rapid diffusion of the antibiotic from the fibrin carrier. High concentrations of cefotaxim could only be maintained locally in the wound exudate for up to 72 hours. In a separate study, Dahners and Funderburk implanted gentamicin-loaded plaster of paris into the tibiae of rabbits with established staphylococcal osteomyelitis. Although they observed clinical and roentgenographic improvements as

compared with nontreated controls, nevertheless, 80 animals treated with the gentamicin-loaded plaster of paris developed culture-positive osteomyelitis. Recently Gerhart et al. evaluated poly(propylenefumarate-co-methylmethacrylate) (PPF-MMA), as a potential biodegradable carrier for antibiotics. Following the subcutaneous implantation of gentamicin- or vancomycin-loaded cylinders of PPF-MMA in rats, high concentrations of each antibiotic were detected locally in the wound exudate while serum antibiotic levels remained low. Although the PPF-MMA appears promising as a potential biodegradable antibiotic carrier, the efficacy of this system remains to be demonstrated in an experimental animal model of osteomyelitis.

In the present application we evaluated biodegradable microspheres of poly(DL-lactide-co-glycolide), containing 30.7 weight percent ampicillin anhydrate, in an experimental osteomyelitis model of the rabbit tibia. In the initial experiment where treatment was initiated immediately following the injection of S. aureus into the medullary canal, a single intramedullary injection of 100 mg of microencapsulated ampicillin effectively prevented the establishment of osteomyelitis in 100 of the animals tested (Table 2). Although a 14 day course of parenteral ampicillin therapy also prevented osteomyelitis in all animals, the total dose of antibiotic administered to these animals (1,050 mg) was 34 times higher than the dose administered to the animals treated locally with the ampicillin-loaded

microcapsules (30.7 mg).

In the second experiment, where antibiotic therapy was delayed for 7 days and was instituted without debridement, a 14 day course of parenteral ampicillin therapy resulted in a 75 treatment failure rate (Table 3). Free unencapsulated ampicillin, implanted locally into the bone, was also ineffective with 71 these animals developing culture-proven osteomyelitis. A single intramedullary application of microencapsulated ampicillin, on the other hand, sterilized the tibiae of 50 significantly reduced the mean number of S. aureus colonies recovered from the tibiae of the other animals in this group. It is noteworthy that all animals treated locally with microencapsulated ampicillin anhydrate survived the duration of the experimental protocol without developing adverse side-effects. In contrast, 6 of 8 (75 parenteral ampicillin died within 1 to 2 weeks of completing their antibiotic therapy. The cause of death in these animals was most likely antibiotic-induced diarrhea resulting from colonization of the normal intestinal flora by Clostridium difficile, a phenomenon that has been previously noted with rabbits receiving parenteral ampicillin therapy.

In the final experiment, where local antibiotic therapy was delayed for 7 days and was instituted in conjunction with debridement, a 100 animals treated with debridement plus microencapsulated ampicillin (Table 4). In contrast, of the 10

animals treated with debridement plus an equivalent dose of unencapsulated ampicillin powder, 70 seen in Figure 5, at 1 hour after implantation of the antibiotics into the medullary canal, the mean serum concentration of ampicillin in the animals receiving unencapsulated ampicillin was approximately 7 times higher ($0.79 \pm .024$ micrograms/ml) than in the group that was treated with microencapsulated ampicillin anhydrate (0.11 ± 0.08 micrograms/ml). This finding suggests that the free unencapsulated drug diffuses rapidly from the site of administration and does not remain localized for a sufficient time interval to eradicate the infection. The fact that 70 animals treated with the unencapsulated form of the drug developed osteomyelitis substantiates this conclusion. The ampicillin-loaded microcapsules/spheres, on the other hand, remain localized at the site of administration thereby continuing to release high concentrations of the antibiotic over time resulting in the elimination of the infecting organisms.

Applicants' experimental studies have demonstrated that a controlled-release and biodegradable antibiotic delivery system was successful for the eradication of a susceptible organism from an osteomyelitic focus when used in conjunction with adequate debridement.

Preparation of Ampicillin Anhydrate Microcapsules

EXAMPLE 8

About 500 g of a 10 wt alcohol) (PVA) was added to a 1-L (liter) resin kettle and cooled to 5°C while being stirred at 650 rpm with a 2.5-in. Teflon turbine impeller driven by a motor and a control unit. A solution consisting of 5 g of 68:32 poly(DL-lactide-co-glycolide) in a mixture of 40 g of dichloromethane and 20 g of acetone was prepared in a separate container and stirred magnetically while in an ice bath. In still another container, 5 g of ampicillin anhydrate was dispersed in 15 g acetone. This mixture was stirred magnetically and then sonicated to achieve uniform dispersion of single ampicillin anhydrate crystals. After sonication, the container was placed in an ice bath, magnetic stirring was continued, and additional acetone was added to give a total of 30 g of acetone. After complete dissolution of the copolymer, the ampicillin-acetone dispersion was added to the copolymer solution. The resulting mixture was stirred magnetically in an ice bath for about 30 minutes or until homogeneous, and it was then added to the reaction flask containing the aqueous PVA solution. The stir rate was reduced from 650 to 500 rpm after the addition was complete. After 15 minutes, the pressure was reduced to 550 torr to begin slow evaporation of the organic solvent (dichloromethane and acetone). The pressure was further reduced to 250 torr. This pressure was maintained for another 18 to 24 hours. The flask was then opened, the suspension was removed, and the microcapsules were separated from the PVA solution by centrifugation. The microcapsules were then washed twice with water, centrifuged, and

washed once more with water and recovered by filtration. The microcapsules were then dried in vacuo and separated into various size fractions by sieving. A free-flowing powder of spherical particles was obtained.

EXAMPLE 9

Dissolve 1.2 g of 50:50 poly(DL-lactide-co-glycolide) in 102 g of methylene chloride. Ampicillin anhydrate (0.8 g) is next added to the stirring copolymer solution. This mixture (dispersion of drug in the copolymer solution) is then placed in a 200-mL resin kettle equipped with a true bore stirrer having a 1.5-inch Teflon turbine impeller driven by a motor. While the mixture is stirring at 700 to 800 rpm, 48 mL of 100 centastoke (cSt) silicone oil is pumped into the resin kettle to cause the poly(DL-lactide-co-glycolide) to coacervate and coat the dispersed ampicillin anhydrate particles. After the silicone oil is added to the resin kettle, the contents of the kettle are poured into heptane to harden the microcapsules/spheres. After stirring in the heptane for 2 hours, the microcapsules/spheres are collected on a funnel and dried. A free-flowing powder of spherical different sized particles is obtained.

In Vitro Characterization of Microcapsules/spheres

The core loadings of microcapsules/spheres comprising [¹⁴C]-ampicillin anhydrate and DL-PLG were measured by liquid scintillation counting. The core loading of microcapsules/spheres

consisting of unlabeled ampicillin anhydrate and some radiolabeled ampicillin anhydrate and DL-PLG was measured by using a microbial assay. In the former instance, microcapsules/spheres (about 15 mg) were solubilized in 1 mL of 0.5 N dimethyl dialkyl quarternary ammonium hydroxide in toluene (Solune-350) at 55°C for 2 to 4

hours. Then, 14 ml of scintillation cocktail (1,4-bis[2-(5-phenyloxazolyl)] benzene (PPO/POPOP) in toluene) was added, and the radioactivity was measured with a liquid scintillation spectrometer. In the latter instance, microcapsules/spheres (about 15 mg) were placed in 5 mL of methylene chloride. Following dissolution of the DL-PLG excipient, the insoluble ampicillin anhydrate was extracted from the methylene chloride with four volumes of sterile 0.1 M potassium phosphate buffer (pH 8.0). These aqueous extracts were then assayed for the antibiotic using Sarcina lutea ATCC 9341 (American Tye Culture Collection, Rockville, MD) and the agar-diffusion microbial assay previously described in the literature by Kavanagh, F. (ed.) Antibiotic Substances in Analytical Microbiology, Vol. II, 1972.

The in vitro release kinetics of [¹⁴C]-ampicillin anhydrate microcapsules/spheres was determined following the placement of 30 mg of microcapsules in an 8-ounce bottle. The release study was initiated by the addition of 50 mL of receiving fluid consisting of 0.1 m potassium phosphate buffer (pH 7.4). The bottle was then sealed and placed in an oscillating (125 cycles/ minutes) shaker bath maintained at 37°C. Periodically, a

3-ml aliquot of the receiving fluid was removed for assay and replaced with a fresh 3-ml aliquot of receiving fluid to maintain a constant volume of receiving fluid throughout the study. The 3-ml aliquots were assayed for drug by liquid scintillation counting using 12 ml Scinti Verse-I (Fisher Scientific Co., Pittsburgh, PA). The cumulative amount of the drug released into the receiving fluid was calculated.

The in vitro release kinetics of unlabeled ampicillin anhydrate microcapsules/spheres was determined in the following manner:

A known amount of ampicillin anhydrate microcapsules/spheres (about 4 mg of microencapsulated ampicillin anhydrate) and 5.0 ml of sterile receiving fluid (0.1 M potassium phosphate buffer, pH 7.4) were added into dialysis tubing. The ends of the tubing were sealed with plastic clamps. The clamped dialysis tubing containing the microcapsules/spheres were placed into a sterile 8-ounce bottle containing 100 ml of sterile receiving fluid (0.1 M potassium phosphate buffer, pH 7.4). The bottle was placed in a shaker bath maintained at 37°C and shaken at 120 cycles per second with about 3-cm stroke. The receiving fluid was previously sterilized in an autoclave for 20 minutes at 121°C. Several dialysis tubing assemblies were prepared for one release study. At Days 1, 2, 4, 7, 10, 13, 15, 18, and 25, one assembly was removed from its receiving fluid and air dried.

After drying the assembly, all particles remaining inside the dialysis tubing were quantitatively transferred to a sterile, glass test tube (16 by 125 mm), 5 ml of methylene chloride were added to dissolve the microcapsules, and the drug was extracted with three 5-ml portions of sterile 0.1 M potassium phosphate buffer (pH 8.1). The extraction and preparation of the sample (along with controls) was performed using the procedures for core-loading analysis as discussed above in the extracted samples and controls using the microbiological assay. Knowing the amount of microencapsulated drug initially placed in the dialysis tubing and the amount of drug remaining in the dialysis tubing after incubation with receiving fluid, the amount of drug released was determined by calculating the difference between them.

In Vivo Release Profiles of Ampicillin from Microcapsules/spheres

The rate and duration of release of ampicillin anhydrate from the microcapsules/spheres were determined in vivo in rats. In one experiment, about 50- to 80-mg doses of microencapsulated and unencapsulated ampicillin anhydrate were sterilized in disposable syringes with a 2.0- or 2.5-Mrad dose of gamma radiation at dry-ice temperature. The sterile microcapsules/spheres and unencapsulated [^{14}C]-ampicillin anhydrate were then suspended in about 2.0 mL of an injection vehicle comprising 2 wt percent of commercially available carboxymethyl cellulose (Type 7LF, Hercules Inc., Wilmington, DE) and 1 wt percent Tween 20 (ICI Americas Inc., Wilmington, DE) in sterile water and autoclaved at 121°C for 15 minutes. The microcapsules/spheres were administered subcutaneously into the mid-back region of lightly anesthetized (ether), male Sprague-Dawley rats. The rats were fed standard laboratory food and water ad libidum and were housed in individual stainless steel cages fitted with metabolism funnels and screens that separated and collected the feces and urine. The urine from each rat was collected, weighed, and analyzed for [^{14}C]-content by liquid scintillation counting. The actual doses of microcapsules/spheres or unencapsulated drug administered to each rat was determined after injection by measuring the amount of drug residue in each syringe by liquid scintillation counting. The amount of radioactivity excreted daily by each rat was normalized by the dose of microencapsulated or unencapsulated ampicillin anhydrate

that each rat actually received. This result was then plotted as a function of time.

In a second experiment, unlabelled ampicillin anhydrate microcapsules/spheres were tested in rats. The rats were administered the microcapsules/spheres in the same manner as that described in the first experiment. The microbiological assay described above was used to determine the amount of ampicillin in the serum of these rats.

In Vivo Efficacy Evaluation of Microcapsules/spheres

Experiments to evaluate the efficacy of prototype microcapsules/spheres in vivo were performed in 250- to 300-g male, Walter Reed strain, albino rats that were anesthetized with sodium pentobarbital. The right hind leg was razor-shaved, scrubbed with Betadine (The Purdue Frederick Co., Norwalk, CT), and swabbed with 70length and 1 cm deep was made in the thigh muscle and contaminated with 0.2 g of sterile dirt. The muscles were traumatized by uniformly pinching them with tissue forceps, and then the wounds were inoculated with known quantities of Staphylococcus aureus ATCC 6538P and Streptococcus pyogenes ATCC 19615. All rats were inoculated on the same day of the experiment with the same quantitated bacterial suspension to insure uniform inoculum in all wounds. The artificially contaminated wounds were treated within 1 hour by layering sterile, pre-weighed amounts of microencapsulated antibiotic directly on the wounds. Control

groups consisted of animals with wounds that either received no therapy, were overlaid with placebo (unloaded) microcapsules/spheres, or were treated with locally applied, powdered unencapsulated ampicillin anhydrate. Following treatment, all wounds were sutured closed with 3-0 black silk.

Three groups of 20 rats each were used in an efficacy experiment to evaluate Microcapsules/spheres A382-140-1 formulated from 70:30 DL-PLG. In this experiment, a group of animals with wounds overlaid with 0.5 g of unloaded microcapsules/spheres was substituted for the untreated (no therapy) group evaluated in each succeeding dose-response experiment. In addition, a group of 20 rats treated with 0.5 g of ampicillin anhydrate microcapsules/spheres per wound, and a group of 20 rats treated with 120 mg of locally applied uncapsulated ampicillin anhydrate powder per wound were evaluated. Five animals from each group were sacrificed at 2, 6, 8, and 14 days and evaluated for the presence of ampicillin in the serum and tissue and for the presence of infection.

Two dose-response experiments were performed in which Microcapsules/spheres A681-31-1, formulated from 70:30 DL-PLG, and Microcapsules/spheres B213-66-1S, formulated from 53:47 DL-PLG were evaluated. Seven groups of 15 rats each were treated with the doses of microcapsules shown in Table I. Each experiment included an additional group of 15 rats which remained untreated.

In dose-response Experiment I, five animals from each group were sacrificed at 2, 7, and 14 days and evaluated for ampicillin levels and number of bacteria present per gram of tissue at each wound site. Serum ampicillin levels were assayed at 2, 4, 7, and 14 days. In dose-response Experiment II, five animals from each group were sacrificed at 7, 14, and 21 days and evaluated for ampicillin levels and number of bacteria present per gram of tissue. Serum ampicillin levels were determined at 2, 7, 14, and 21 days.

Microcapsules/spheres in a 45 to 106 micron size range made by the phase-separation process were evaluated in these experiments. The ampicillin anhydrate content of the microcapsules/spheres (core loading), batch number, and ampicillin anhydrate equivalent for each dose of microcapsules/spheres are shown in Table 1.

In all experiments, bacterial counts were performed on homogenized, preweighed tissue that had been aseptically removed from the wound sites. Serial dilutions of the homogenized tissue specimens were plated on sheep blood agar. Colonies of Staphylococcus aureus could be easily differentiated from Streptococcus pyogenes on the basis of colonial morphology. Tissue from varying distances around the wound site and serum removed by cardiac puncture were assayed for antibiotic content. This was accomplished by placing discs saturated with known

quantities of serum or tissue homogenates on the surface of Mueller-Hinton agar which had been previously seeded with standardized amounts of Sarcina lutea ATCC 9341. Following incubation at 37°C, inhibition zones were measured. Freshly diluted stock solutions containing known quantities of ampicillin anhydrate served as standards. Diameters of the inhibition zones were converted to antibiotic concentrations using standard curves generated by plotting the logarithm of the drug concentration against the zone diameters.

TEST RESULTS

Microcapsule/spheres In Vitro Evaluation

Ampicillin anhydrate was microencapsulated with DL-PLG excipient. DL-PLG is a biocompatible aliphatic polyester that undergoes random, nonenzymatic, hydrolytic scission of the ester linkages under physiological conditions to form lactic acid and glycolic acid. These hydrolysis products are readily metabolized.

The purpose of the DL-PLG is to control the release of the ampicillin anhydrate from the antibiotic microcapsule/spheres formulation and to protect the reservoir of ampicillin anhydrate from degradation before it is released from the microcapsules/spheres. Two DL-PLG excipients were used in this study. One DL-PLG had a lactide-to-glycolide mole ratio of 70:30 and the other, 53:47. The 53:47 DL-PLG biodegrades faster than the 70:30 DL-PLG because of its higher glycolide content.

A phase-separation microencapsulation process afforded microcapsules/spheres in yields of better than 95%. The microencapsulated ampicillin anhydrous product was a fine, free-flowing powder. The microcapsules/spheres are relatively spherical in shape, but have puckered regions. Although these puckered regions exist, the polymer coating was continuous, and there was no evidence of any fractures or pinholes on the surfaces of the microcapsules. Moreover, the photomicrograph obtained by scanning electron microscopy of ampicillin anhydrate microcapsules did not show any evidence of free unencapsulated ampicillin anhydrate crystals either among the microcapsules or protruding through the surface of the microcapsules.

The drug content (core loading) of the ampicillin anhydrate microcapsule/sphere formations was measured to assess how much ampicillin anhydrate was incorporated in the microcapsules and to determine the bioactivity of the ampicillin anhydrate after it had been microencapsulated.

In general, the core loading of the 45-to 106 microns size fraction was similar to the theoretical core loading. The core loading of a few batches of [^{14}C]-ampicillin anhydrate microcapsules/spheres was determined by microbial assay as well as by radioassay. Within experimental error, both assays gave similar results. This indicates that the ampicillin anhydrate was not inactivated during the microencapsulation process. Also, the

core loading of ampicillin anhydrate microcapsules/spheres was determined by the microbial assay to determine the effect of 2.5 Mrad of gamma radiation on the microencapsulated drug. The radiation did not inactivate the drug because the core loading remained the same. For instance, 19.3 spheres with 70:30 DL-PLG assayed as 19.0 irradiation and 11.0 DL-PLG assayed as 11.4 irradiated unencapsulated and microencapsulated drug were also checked by thin layer chromatography. Irradiated and nonirradiated samples chromatographed the same, again indicating that no degradation of the drug was caused by gamma radiation at a dose of 2.5 Mrad.

In vitro release measurements were used to identify an ampicillin anhydrate microcapsule/sphere formulation that would release all of its drug at a controlled rate over a period of two weeks. The formulation that displayed the desired in vitro release kinetics were microcapsules/spheres with diameters of 45 to 106 microns consisting of about 10 wt percent ampicillin anhydrate (Bristol Laboratories, Syracuse, NY) and microcapsules/spheres with diameters of 10 to 100 microns consisting of about 35 wt percent ampicillin anhydrate (Wyeth Laboratories, West Chester, PA) and about 65 wt percent 53:47 DL-PLG. Figures 3 and 4 show the in vitro release profiles of two samples of these prototype microcapsules. The microcapsules released a desirable initial burst of drug, representing about 30% of the drug. The remaining drug was then released at a slower controlled rate.

The in vitro release profile of sterilized (2.5 Mrad), 17.6 compared with the release profiles of sterilized (2.0 Mrad), 9.6 and 7.8 DL-PLG (Figure 3).

Microcapsule/sphere In Vivo Evaluation

Pharmacokinetic studies were performed with unencapsulated ampicillin anhydrate and the same samples of microcapsules that were tested in vitro, as previously described.

As shown in Figures 3 and 4, the unencapsulated drug as well as the microcapsules/spheres showed a fast release of drug during Day 1. By Day 4, the amount of ampicillin found in the urine or serum of animals dosed with the unencapsulated drug was below the level of detection of the assay. On the other hand, the microcapsule/sphere formulations maintained an elevated level of drug in the urine or serum for extended periods. Both samples of microcapsules/spheres made with the 53:47 DL-PLG had similar release profiles and released drug for about two weeks. As illustrated in Figure 5, the microcapsules/spheres prepared with 70:30 DL-PLG released drug for at least four weeks. The results of these pharmacokinetic studies corroborate results of the in vivo release studies described. The 53:47 microcapsules/spheres closely meet the desired target duration of release of two weeks.

The slow rate of ampicillin release from the 70:30 microcapsules/spheres, as shown in Figure 5, may be undesirable because a low level of ampicillin anhydrate released over a long

period may provide favorable conditions for the development of drug-resistant bacterial strains. This slower release of drug could be attributed to the slower biodegradation rate of the 70:30 DL-PLG, where the water-soluble ampicillin anhydrate remained trapped inside the hydrophobic DL-PLG excipient until the excipient biodegraded completely. More specifically, for microcapsules/spheres prepared with either the 70:30 or 53:47 DL-PLG, one could speculate that the release of drug is due to diffusion of the drug through water-filled pores, pores that enlarge as more and more drug is released and as the DL-PLG bioerodes.

However, all ampicillin anhydrate microcapsules/spheres formulated effectively reduced bacterial counts in contaminated wounds. The most dramatic observation was the rapid elimination of Streptococcus pyogenes. Streptococcus pyogenes was present in 90 from microcapsule/sphere-treated wounds within 48 hours. All three of the microcapsule/sphere batches evaluated were equally successful in eliminating this organism within two days. At 7 days Staphylococcus aureus remained in all treated wounds; however, compared to untreated controls, the bacterial count per gram of tissue decreased by at least 2 log₁₀ between Days 2 and 7.

This reduction was not observed in untreated controls. In the efficacy evaluation of microcapsules/spheres A382-140-1, wounds treated with unloaded DL-PLG microcapsules, as well as those treated with topical unencapsulated ampicillin anhydrate, remained

infected at 14 days with $> 10^5$ organisms per gram of tissue; whereas, 60 ampicillin anhydrate were sterile. The wounds of the remaining 4010^3 organisms per gram of tissue. By 14 days, regardless of the dose administered (0.5-0.05 g), all wounds treated with microcapsule/sphere sample A681-31-1 were sterile; whereas, all untreated wounds remained infected with $> 10^5$ organisms per gram of tissue. At 14 days, all wounds treated with 0.15 g of microcapsules/spheres B213-66-1S were sterile, however, 5.7×10^2 Staphylococcus aureus per gram of tissue were counted in the wounds of one animal treated with a 0.25-g dose of encapsulated ampicillin anhydrate. This failure was attributed to an abscess around a suture on the wound surface. All wounds treated with 0.15 g of microcapsules/spheres (B213-66-1S) were sterile; however, in the group treated with a 0.05-g dose of microcapsules/spheres, one wound remained contaminated with 3.6×10^4 Staphylococcus aureus per gram of tissue. The untreated control animals, evaluated in parallel with the microcapsule/sphere-treated groups, averaged 1.4×10^5 Staphylococcus aureus per gram of tissue.

Serum levels of drug were dependent upon the ampicillin anhydrate reservoir present inside the microcapsules/spheres (core loading), the dose, and the ampicillin release characteristics. Administration of 0.25 g of Microcapsules/spheres A681-31-1, which contained a 45.25 mg ampicillin reservoir per wound, maintained a serum ampicillin level of 8.0 ± 7.3 microgram/milliliter for up to

4 days post-treatment. A dose twice that amount (90.50 mg ampicillin equivalent) maintained detectable serum ampicillin for up to 7 days post-treatment at a serum ampicillin concentration of 15.95 ± 5.0 microgram/milliliter for the first 4 days. Serum ampicillin was not detected in animals whose wounds were treated with microcapsule/sphere doses containing an ampicillin equivalent of 28.50 mg or less. Even though serum ampicillin was not detected in any animal at 14 days, the tissue levels at this time were above the minimal inhibitory concentrations required to kill both infecting organisms in all animals treated with microencapsulated ampicillin anhydrate. This was true with microcapsule/sphere doses as low as 0.05 gram per wound. Even though serum ampicillin was not detected, microbial bioassay for ampicillin in tissue removed from wounds treated with 0.05 gram of microcapsules/spheres (A681-31-1) contained a mean (n=5) ampicillin level of 54, 70, and 21 micrograms/gram of tissue at 2, 7, and 14 days, respectively. Because the minimal inhibitory concentrations of ampicillin required to kill 95 of Staphylococcus aureus and 97 pyogenes is 0.5 and 0.05 micrograms/milliliter, respectively, it is a reasonable assumption that a more than adequate therapeutic amount of drug was present at the wound site throughout the two-week treatment period.

In vitro release studies performed on microcapsules/spheres formulated with 70:30 DL-PLG (A382-140-1 and A681-31-1) showed drug release at an efficacious rate over two weeks, but also at a slower rate for an additional 50 days. The continued

release of low amounts of antibiotic in wounds after two to three weeks is undesirable because of the potential to provide favorable conditions for the emergence of ampicillin resistant organisms in wounds which might harbor small numbers of bacteria. Therefore, to reduce or eliminate drug trailing, microcapsules/spheres were reformulated by encapsulating ampicillin anhydrate within the faster biodegrading polymer 53:47, DL-PLG (sample B213-66-1S), in vitro release profiles showed a release of 85 to 92 within two weeks. On the seventh day following treatment of wounds with 0.15 gram of Microcapsules/spheres B213-66-1S, a mean (n=5) of 162.5 g of ampicillin per gram of tissue was quantitated. In vitro release studies suggest that this amount drops rapidly in the second week so that by 14 days marginal killing concentrations are present. In vivo analysis of tissue removed from wounds treated 15 days previously with 0.25 gram of these microcapsules/spheres contained < 1.9 micrograms/gram of ampicillin per gram. Although <0.22 micrograms/gram of ampicillin was detected in wounds treated with 0.15 gram, it was unusual to detect any ampicillin at 14 days in tissue from wounds treated with 0.05 gram per wound. At 21 days post-treatment, ampicillin was not detected in any of the wounds.

No serum levels of ampicillin were detected in any of the rats treated with Microcapsules/spheres B213-66-1S. This was expected because lower doses (ampicillin equivalent) were administered. (Table 1).

B. Cefazolin (CZ) microspheres. The CZ microspheres used in these studies were produced by Southern Research Institute, Birmingham, AL. The microspheres consisted of 77.8 weight % copolymer (50:50 molar ratio of lactide to glycolide) with a core leading dose of 22.2 weight % cefazolin. The size of the microspheres ranged from 90 to 355 um in diameter and they were sterilized with 2.7 Mrad of gamma radiation. In vitro release kinetic studies showed that approximately 20% of the cefazolin was released from the microspheres within 6 hours, with the remainder of antibiotic release extending over a period of 15 days.

Rat wound infection model. Experimental wounds were surgically created in the paraspinous muscles of Sprague-Dawley rats following induction of anesthesia with ketamine and xylazine. Sterile sand (100 mg) was implanted into the wound site to simulate a foreign body and the wounds were inoculated with 5×10^6 CFU each of Staphylococcus aureus ATCC 27660 and Escherichia coli ATCC 25922. The minimum inhibitory concentration (MIC) of cefazolin for each of these organisms was 4 ug/ml and 2 ug/ml, respectively. The animals were then randomly distributed in 6 groups. Groups A, B, and C (6 rats per group) received local antibiotic therapy with 50 mg, 250 mg, or 500 mg of CZ microspheres, respectively. The microspheres were applied directly to the wounds and care was taken to achieve a relatively uniform distribution of the drug throughout the wound site. Group D (6 rats) received local antibiotic therapy with 110 mg of CZ powder. This dose was equivalent to the core-

loading dose of cefazolin contained in 500 mg of CZ microspheres used to treat the Group C animals. Group E (6 rats) received systemic antibiotic therapy with cefazolin (30 mg/kg) which was administered as a single intramuscular bolus immediately after bacterial contamination of the wounds. Group F (3 rats) served as controls and received no antibiotic therapy. The wounds were then closed with surgical staples and the animals were returned to their cages. On postoperative day # 28, the rats were euthanized and tissue was obtained from each wound for quantitation of surviving bacteria. The tissue was weighed, homogenized, and serial 10-fold dilutions were prepared and plated on blood agar. The number of bacteria recovered from each wound was quantitated and expressed as CFFU/g tissue.

Rabbit fracture-fixation model. This study was conducted in two segments and was designed to evaluate the effect of early as well as delayed local antibiotic therapy for the prevention of infection in experimental fractures. In segment I, open fractures were created in the right tibiae of New Zealand White rabbits after induction of anesthesia with ketamine and xylazine. The fractures were then inoculated with 0.5 ml of S. aureus ATCC 27660 (2.0×10^7 CFU/ml). Within 30 minutes following bacterial contamination, the animals were randomly distributed in 5 groups. Group A (8 rabbits) received local antibiotic therapy with 300 mg of cefazolin microspheres which was applied directly to the fracture site and the deep musculature. Group B (8 rabbits) received local antibiotic therapy

with an equivalent dose of CZ powder. Group C (8 rabbits) received systemic antibiotic therapy with cefazolin (25 mg/kg/day) for 7 days. Groups D and E (4 rabbits per group) served as controls and received either local application of placebo microspheres (without cefazolin) or no treatment, respectively. The fractures were then reduced and plated with a 4-hole dynamic compression plate. Immediately prior to wound closure, animals in Groups A and B received an additional dose of either CZ microspheres (300 mg) or an equivalent dose of CZ powder, respectively, which was applied directly over the fixation plates and the periosteal tissue. The wounds were then repaired with sutures and the animals were returned to their cages. Blood was obtained within 1 hour and again at 24 hours after treatment from all Group A and B animals for quantitation of serum cefazolin levels which was measured by a microbial inhibition bioassay⁹. Eight weeks later, all surviving animals were euthanized and the tibiae were harvested for bacteriological analysis, the bones were crushed to small pieces with sterile mortar and pestle and saline was added to make a particulate suspension. Serial dilutions were then prepared and streaked on blood agar for bacterial isolation. The number of S. aureus colonies recovered from each specimen was quantitated and expressed as CFU/g of bone.

In segment II, fractures were created in the right tibia of 29 rabbits and contaminated with S. aureus as described above. After a 2 hour delay, the animals were randomly distributed in 3 groups.

Group A (10 rabbits) received local antibiotic therapy with 600 mg of CZ microspheres. Group B (10 rabbits) received local antibiotic therapy with an equivalent dose of CZ powder. Group C (9 rabbits) served as controls and received no treatment. The fractures were then reduced, plated, and the wounds were closed with sutures. Eight weeks later, the surviving animals were euthanized and the tibiae were harvested and processed for isolation of bacteria as described above.

Results

Rat wound infection model. Table 5 shows the effect of local versus systemic cefazolin therapy on the contamination rate in rat soft-tissue wounds at 28 days postinfection. Local antibiotic therapy with CZ microspheres, in doses ranging from 50 to 500 mg per wound, was highly effective for eliminating both organisms from the wounds. The maximum effect was achieved in the Group C animals who received the highest dose of CZ microspheres (500 mg) where E. coli and S. aureus were eliminated from 100% of the wounds. Even at the lowest dose used (50 mg/wound), 4 of 6 wounds were rendered completely sterile. Local antibiotic therapy with free CZ powder sterilized the wounds in 5 of 6 (83%) animals. In contrast, systemic administration of cefazolin (30 mg/kg failed to sterilize the wounds in any of the 6 Group E animals tested.

Rabbit fracture-fixation model. Table shows the results of the clinical and bacteriological findings at 8 weeks in 25 surviving rabbits when local or systemic antibiotic therapy with cefazolin was initiated within 30 minutes following bacterial contamination of the fractures. Deep infection, defined as the presence of pus on the fixation plate or in the deep tissues, was noted in 6 of the 7 (86%) control animals in Group D (placebo microspheres) and group E (no treatment). Cultures of the tibiae from all 7 controls were positive for S. aureus. Of the 5 surviving Group animals who received a 1 week course of systemic cefazolin therapy, deep infection was noted in 3 cases and S. aureus was recovered from the bones of 4 of the 5 animals. In contrast, no clinical evidence of infection was detected in any of the 7 Group A animals who received an equivalent local dose of free CZ powder. Cultures of the tibiae were sterile in 6 of (86%) Group A and 5 of 6 (83%) Group B animals, respectively. There was a statistically significant difference in the mean log S. aureus counts of the Group A and Group B animals and all other groups by analysis of variance ($p < 0.05$). The mean log S. aureus counts for Group C was also significantly different from all groups with the exception of Group E (no treatment).

Table 7 shows the results of the clinical and bacteriological findings at 8 weeks in 23 surviving rabbits when local antibiotic therapy was delayed for 2 hours following bacterial contamination of the fractures. Clinical evidence of infection was present in 5 of 7 (71% control animals in Group C and cultures of the tibiae yielded S. aureus in all 7 cases. Of the 8 animals in Group B who received local antibiotic therapy with Cz powder, deep infection was noted in 4 animals and S. aureus was received in 6 of 8 (75%) cases. In contrast, none of the 8 animals in Group Aa (CZ microspheres) developed clinical infections and cultures of the tibiae were sterile in all cases. One way analysis of variance showed a statistically significant difference in the mean log S. aureus counts between Groups A and B ($p = 0.0014$); Groups A and C ($p < 0.0001$); and Groups B and C ($p = 0.0269$).

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Applicants have developed microencapsulated antibiotics for the local treatment of contaminated surgical and traumatic wounds. Preliminary studies have shown that local application of biodegradable antibiotic microspheres to experimental wounds that were contaminated with resistant bacteria was extremely effective for prevention of wound infection. This success is attributed to the significantly higher local tissue antibiotic levels that can be achieved at the wound site with direct local application of microencapsulated antibiotics as compared to conventional systemic antibiotic dosing. The findings of the experimental studies are summarized below:

1. Ampicillin microspheres effectively prevented infection in 8/11 (73%) animals whose wounds were inoculated with an ampicillin-resistant strain of *s. aureus* (MIC = 750 ug/ml). Systemic ampicillin failed in 9/9 (100%) cases.

2. Cefazolin microspheres effectively prevented infection in 5/6 (83%) animals whose wounds were inoculated with a methicillin-resistant strain of *S. aureus* which was also resistant to cefazolin (MIC = 64 ug/ml). Systemic cefazolin failed in 5/6 (83%) cases.

3. It is preferred that a initial release (burst) of the encapsulated antibiotic occur within the first day and the remaining antibiotic be released over the next 2 to 3 weeks.

EXPERIMENTAL DESIGN FOR RAT SOFT-TISSUE WOUND INFECTION MODEL

Experimental surgical wounds were created in the paraspinous muscle of anesthetized Sprague Dawley rats, each weighing between 450 to 550 grams. The wounds were then contaminated with 100 mg of sterile sand as an infection-potentiating agent. The wounds were then inoculated with 5×10^6 CFU of *S. aureus* ATCC 33593. This is a methicillin-resistant strain of *S. aureus* which is also resistant to cefazolin (MIC = 64 ug/ml). The animals were then assigned to the following treatment groups:

Group A (n = 6): 500 mg of cefazolin (CZ) microspheres was applied directly to the wounds. This dose contained 110 mg of cefazolin equivalent.

Group B (n = 6): 110 mg of free CZ powder was applied directly to the wounds.

Group C (n = 6): This group received intramuscular injections of CZ (30 mg/kg/day) at 8 hour intervals for 7 consecutive days.

Group (n = 3): This group served as controls and did not receive any antibiotic therapy.

The wounds were then closed with surgical staples and the animals were returned to their cages for the next 5 weeks. At that time, the animals were humanely euthanized and tissue was removed from the wounds and cultured for the presence of bacteria. The bacteriological data are presented in Table 8.

VIII. UTILITY

Successful controlled release of bioactive ampicillin anhydrate was achieved in vitro and in vivo. The prototype microcapsules/spheres effectively controlled or eliminated Staphylococcus aureus and Streptococcus pyogenes from infected wounds in rats. Additionally, the formulation would be effective in the treatment of all bacterial infections caused by organisms sensitive to the antibiotic encapsulated including but not limited to Enterobacteriaceae; Klebsiella sp.; Bacteroides sp.; Enterococci; Proteus sp.; Streptococcus sp.; Staphylococcus sp.; Pseudomonas sp.; Neisseria sp.; Peptostreptococcus sp.; Fusobacterium sp.; Actinomyces sp.; Mycobacterium sp.; Listeria sp.; Corynebacterium sp.; Propionibacterium sp.; Actinobacillus sp.; Aerobacter sp.; Borrelia sp.; Campylobacter sp.; Cytophaga sp.; Pasteurella sp.; Clostridium sp.; Enterobacter aerogenes; Peptococcus sp.; Proteus vulgaris; Proteus morganii; Staphylococcus aureus; Streptococcus polygenes; Actinomyces sp.;

Campylobacter fet and Legionella pneumoph. Results indicate that optimal microcapsules/spheres should exhibit a programmed release of an appropriate concentration of antibiotic over about a 14 day to about a 6 week time period after which time the microcapsule/sphere should biodegrade, leaving no trace of drug or excipient.

PHASE II

This illustrative phase of this invention relates to a novel pharmaceutical composition, a microcapsule/sphere formulation, may contain a pharmaceutically-acceptable adjuvant that comprises an antigen encapsulated within a biodegradable polymeric matrix such as poly

1 (DL-lactide-co-glycolide) (DL-PLG), wherein the relative ratio between the
2 lactide and glycolide component of the DL-PLG is within the range of 90:10 to
3 0:100, and its use, as a vaccine, in the effective pretreatment of animals
4 (including humans) to prevent intestinal infections caused by a virus or
5 bacteria. In the practice of this invention, applicants found that the AF/R1
6 adherence factor is a plasmid encoded pilus composed of repeating pilin
7 protein subunits that allows E. coli RDEC-1 to attach to rabbit intestinal brush
8 borders. To identify an approach that enhances the immunogenicity of
9 antigens that contact the intestinal mucosa, applicants investigated the effect of
10 homogeneously dispersing AF/R1 pili within biodegradable microspheres that
11 included a size range selected for Peyer's Patch localization. New Zealand
12 White rabbits were primed twice with 50 micrograms of either
13 microencapsulated or nonencapsulated AF/R1 by endoscopic intraduodenal
14 inoculation. Lymphoid tissues were removed and cellular proliferative
15 responses to AF/R1 and synthetic AF/R1 peptides were measured in vitro.
16 The synthetic peptides represented possible T and/or B cell epitopes which
17 were selected from the AF/R1 subunit sequence using theoretical criteria. In
18 rabbits which had received nonencapsulated AF/R1, Peyer's Patch cells
19 demonstrated slight but significant proliferation in vitro in response to AF/R1
20 pili but not the AF/R1 synthetic peptides. In rabbits which had received
21 microencapsulated AF/R1, Peyer's Patch cells demonstrated a markedly
22 enhanced response to AF/R1 and the synthetic peptides. Cells from the
23 spleen and mesenteric lymph nodes responded similarly to AF/R1 pili in both
24 groups of animals, while there was a greater response to the synthetic peptide

1 AF/R1 40-55 in rabbits that had received microencapsulated AF/R1. These
2 data demonstrate that microencapsulation of AF/R1 potentiates the mucosal
3 cellular immune response to both the native protein and its linear peptide
4 antigens.

5 A primary mucosal immune response, characterized by antipilus
6 IgA, follows infection of rabbits with E. coli RDEC-1. However, induction of
7 an optimal primary mucosal response by enteral vaccination with pilus antigen
8 depends on immunogenicity of pilus protein, as well as such factors as its
9 ability to survive gastrointestinal tract (GI) transit and to target
10 immunoresponsive tissue. We tested the effect of incorporating AF/R1 pilus
11 antigen into resorbable microspheres upon its ability to induce primary mucosal
12 and systemic antibody responses after direct inoculation into the GI tract.

13 METHODS: rabbits were inoculated with 50 micrograms of AF/R1 pilus
14 antigen alone or incorporated into uniformly sized (5-10 microns) resorbably
15 microspheres (MIC) of poly(DL-lactide-coglycolide). Inoculation was by
16 intra-duodenal (ID) intubation via endoscopy or directly into the ileum near a
17 Peyer's patch via the RITARD procedure (with the cecum ligated to enhance
18 recovery of gut secretions and a reversible ileal tie to slow antigen clearance).
19 ID rabbits were sacrificed at 2 weeks for collection of gut washes and serum.
20 RITARD rabbits were bled and purged weekly for 3 weeks with Co-lyte to
21 obtain gut secretions. Anti-pilus IgA and IgG were measured by ELISA.

22 TABLE 9

23 RESULTS: *pos/test RITARD-PILI RITARD-MIC ID-PILI ID-MIC
24

1	Anti-pilus IgA (fluid)	*7/8	4/8	1/2	0/3
2	Anti-pilus IgG (serum)	0/8	3/8	0/2	1/3

3 Native pilus antigen led to a mucosal IgA response in 7/8 RITARD
 4 rabbits. MIC caused a similar response in only 4/8, but the groups were not
 5 statistically different. MIC (but not pili) induced some systemic IgG responses
 6 (highest in animals without mucosal responses). Results in rabbits inoculated
 7 ID were similar for pili, but no mucosal response to ID-MIC was noted.

8 SUMMARY: Inoculation with pilus antigen produces a primary mucosal IgA
 9 response. Microencapsulation does not enhance this response, although the
 10 antigen remains immunogenic as shown by measurable mucosal and some
 11 strong serum responses. It must be determined whether priming with antigen
 12 in microspheres can enhance secondary responses.

13 B CELL EPITOPE DATA

14 Materials and Methods

15 CFA/I PURIFICATION- INTACT CFA/I pili were purified from
 16 H10407 (078:H-) as described by Hall et al, (1989) [20]. Briefly, bacteria
 17 grown on colonization factor antigen agar were subjected to shearing, with the
 18 shearate subjected to differential centrifugation and isopycnic banding on
 19 cesium chloride in the presence of N-lauryl sarkosine. CFA/I were dissociated
 20 to free subunits in 6M guanidinium HCl, 0.2 M ammonium bicarbonate (2
 21 hr, 25°), passed through an ultrafiltration membrane (Amicon XM 50 stirred
 22 cell, Danvers, MA), with concentration and buffer exchange to PBS on a YM
 23 10 stirred cell (Amicon). Examination of dissociated pili by electron
 24 microscopy demonstrated a lack of pilus structure.

1 **Protein Sequencing-** The primary structure of CFA/I has been
2 determined by protein sequencing techniques (Klemm, 1982) and through
3 molecular cloning methods (Karjalainen, et al 1989) [21]. In these tw studies
4 there was agreement in all but two of the 147 amino acid residues (at positions
5 53 and 74). To resolve the apparent discrepancies, CFA/I was enzymatically
6 digested in order to obtain internal amino acid sequence. Trypsin or *S. aureus*
7 V8 protease (sequencing grade, Boehringer Mannheim) was incubated with
8 CFA/I at a 1:50 w:w ratio (Tris 50 mM, 0.1% SDS, pH 8.5 for 16h at 37°
9 (trypsin) or 24°C (V8)). Digested material was loaded onto precast 16%
10 tricine SDS-PAGE gels (Schagger and von Jagow, 1987) (Novex, Encinitis,
11 CA) and run following manufacturers instructions. Separated samples were
12 electrophoretically transferred to PVDF membranes (Westrans, Schleicher and
13 Schuell, Keene, NH) following Matsiduria (1987) using the Novex miniblott
14 apparatus. Blotted proteins were stained with Rapid Coomassie stain
15 (Diversified Biotech, Newton Centre, MA). To obtain the desired fragment
16 containing the residue of interest within a region accessible by automated gas
17 phase sequencing techniques, molecular weights were estimated from standards
18 of molecular weights 20,400 to 2,512 (trypsin inhibitor, myoglobin, and
19 myoglobin cyanogen bromide fragments; Diversified Biotech) using the
20 corrected molecular weights for the myoglobin fragments as given in Kratzin et
21 al., (1989) [22]. The estimated molecular weights for the unknown CFA/I
22 fragments were compared to calculated molecular weights of fragments as
23 predicted for CFA/I from the sequence of CFA/I as analysed by the
24 PEPTIDESORT program of a package developed by the University of

1 Wisconsin Genetics Computer Group. Selected fragments were cut from the
2 PVDF emembrane and subjected to gas phase sequencing (Applied Biosystem
3 470, Foster City, CA).

4 Monkey Immunization- Three rhesus monkeys (*Macaca mulatta*) were
5 injected intramuscularly with 250 ug of dissociated CFA/I in complete
6 Freund's adjuvent and subsequently with two injections of 250 ug of antgen in
7 incomplete Freund's adjuvent at weekly intervals. Blood was drawn three
8 weeks after primary immunization.

9 Peptide Synthesis- Continuous overlapping octapeptides spanning the
10 entire sequence CFA/I were synthesized onto polyethylene pins by the method
11 of Geysen et al. [16], also known as the PEPSCAN procedure. Derivitized
12 pins and software were purchased from Cambridge Research Biochemicals
13 (Valley Stream, NY). Fmoc-amino acid pentafluorophenyl esters were
14 purchased from Peninsular Laboratories (Belmont, CA),
15 1-hydroxybenzotriazole monohydrate (HYBT) was purchased from Aldrich,
16 and reagent grade solvents from Fisher. To span the entire sequence of CFA/I
17 with a single amino acid overlap of from one peptide to the next, 140 total
18 pins were necessary, with a second complete set of 140 pins synthesized
19 simultaneously.

20 ELISA procedure- Sera raised in monkeys to purified dissociated pili
21 were incubated with the pins in the capture ELISA assay of Geysen et al., [16]
22 with the preimmune sera of the same animal tested at the same dilution
23 simultaneously with the duplicate set of pins. Dilution of sera used on the pins

1 was chosen by initial titration of sera by standard ELISA assay and immunodot
2 blot assay against the same antigen.

3 RESULTS

4 It was essential to utilize the correct sequence of CFA/I in the
5 synthesis of the pins for both T- and B-cell experiments to carry out the studies
6 as planned. At issue were the amino acids at position 53 and 74; incorrect
7 residues at those positions would effect 36 of 138 pins (26%) for T-cell epitope
8 analysis and 30 of 140 pins (21%) for B-cell analysis. To resolve the
9 discrepancy in the literature, purified CFA/I was proteolytically digested
10 separately with trypsin and with *S. aureus* V8 protease (V8). These enzymes
11 were chosen in order to give fragments with the residues of interest (53 and
12 74) relatively near to the N-terminus for automated Edman degradation
13 (preferably 1-15 residues). These digests were separated on tricine
14 SDS-PAGE gels (Fig.24 ^{2.4} A) and molecular masses of fragments estimated. A
15 fragment of 3459 calculated molecular mass is expected from the trypsin digest
16 (corresponding to amino acids 62-94) and a fragment of 5889 calculated
17 molecular mass is expected from the V8 digest (residues 42-95). These
18 fragments were located within each digest (arrows in Fig.24), and a
19 companion gel with four lanes of each digest was run, electrophoretically
20 transferred to PVDF, the bands excised and sequenced. N-terminal sequences
21 of each fragment are given in Fig.24 B. The N-terminal eighteen residues
22 from the trypsin fragment were determined that corresponded to positions
23 62-79 in CFA/I. Position 74, a serine residue was consistent with that
24 determined by Karjalainen et al., (Karjalainen et al., 1989). Nineteen residues

1 of the V8 fragment were determined, corresponding to residues 41-60 of the
2 parent protein. The twelfth residue of the fragment contained an aspartic acid,
3 also consistent with Karjalainen et al., (1989). All other residues sequenced
4 were consistent with those published previously (including residues 1-29, not
5 shown). For the following peptide synthesis were therefore utilized the
6 complete amino acid sequence of CFA/I consistent with Karjalainen et al.,
7 (1989).

8 Sera from monkeys immunized with CFA/I subunits were tested in a
9 modified ELISA assay, with the preimmunization sera tested simultaneously
10 with duplicate pins. Assays results are displayed in Fig. 25. Monkey 2Z2
11 (fig. 2A) responded strongly to six regions of the CFA/I sequence. Peptide 14
12 (the octapeptide 14-21) gave the strongest response with four pins adjacent to
13 it (11, 12, 13, and 15) also appearing to bind significant antibody. The other
14 2Z2 epitopes are centered at peptides 3, 22, 33, 93, and 124. Monkey 184D
15 (Fig. 17B) also responded strongly to peptide 14, although the maximum
16 response was to peptide 13, with strong involvement of peptide 12 in the
17 epitope. Additional epitopes recognized by 184d were centered at peptides 22,
18 33, 66, and 93. The third monkey serum tested, 34, responded to this region
19 of the CFA/I primary structure, both at peptides 1, 12 and weakly at 14.
20 Two other epitopes were identified by 34, centered at peptides 67 and 128.
21 Figure 26 illustrates the amino acids corresponding to the epitopes of CFA/I as
22 defined by the response of these three monkeys aligned with the entire primary
23 structure. The entire antigenic determinants are mapped and areas of overlap

criteria published by Rothbard and Taylor [7]. The sequence numbers of the first amino acid of the predicted segments are shown in Table 1.

Lymphocyte proliferation of monkey spleen cells to CFA/I synthetic peptides. To determine which segments of the CFA/I protein are able to stimulate proliferation of CFA/I immune primate lymphocytes *in vitro*, three Rhesus monkeys were immunized with CFA/I subunits, and their splenic lymphocytes were cultured with synthetic overlapping decapeptides which represented the entire CF/I sequence. Concentrations of peptides used as antigen were 6.0, 0.6, and 0.6 ug/ml. Proliferative responses to the decapeptides were observed in each of the three monkeys (fig.9-11). The majority of the responses occurred at the 0.6 and 0.06 ug/ml concentrations of antigen and within distinct regions of the protein (peptides beginning with residues 8-40, 70-80, and 27-137). A comparison of the responses at the 6.0, 0.6 and 0.06 ug/ml concentrations antigenic peptide for one monkey (2&2) are shown (fig.12-14). Taking into account all concentrations of antigen tested, spleen cells from monkey 184D demonstrated a statistically significant response to decapeptides beginning with CFA/I amino acid residues 3, 4, 8, 12, 15, 21, 26, 28, 33, 88, 102, 10, 133, 134, and 136 (fig.27). Monkey 34 had a significant response to decapeptides beginning with residues 24, 31, 40, 48, 71, 72, 77, 78, 80, 87, and 102, 126 and 133 (Fig.28); monkey 2Z2 responded to decapeptides which began with residues 4, 9, 11, 12, 13, 14, 15, 16, 17, 20, 27, 35, 73, 79, 18, 127, 129, 132, and 133 (fig.27). Peptides beginning with amino acid residues 3 through 2 were synthesized with either a glutamic acid or an asparagine substituted for the aspartic acid residue at

1 position twelve to prevent truncated peptides. The observed responses to
2 peptides beginning with residue 8 (monkey 184d), and residues 9, 11, 12
3 (monkey 2Z2) occurred in response to peptides that had the glutamic acid
4 substitution. However, the observed responses to peptides beginning with
5 residue 3, 4, and 12 (monkey 184D), as well as residue 4 (monkey 2Z2)
6 occurred in response to peptides that had the asparagine substitution. Monkey
7 34 did not respond to any of the peptides that had the substitution at position
8 twelve. All other responses shown were to the natural amino acid sequence of
9 the CFA/I protein. Statistical significance was determined by comparing the
10 cpm of quadruplicate wells cultured with the CFA/I peptides to the cpm of
11 wells cultured with the CFA/I peptides to the cpm of wells cultured with a
12 control peptide.

13 Analysis of decapeptides that supported proliferation of lymphocytes
14 from CFA/I immune animals. Of the 39 different peptides that supported
15 proliferative responses, thirty contained a serine residue, 19 contained a serine
16 at either position 2, 3, or 4, and nine had a serine specifically at position 3.
17 Some of the most robust responses were to the peptides that contained a serine
18 residue at the third position. The amino acid sequence of four such peptides is
19 shown in Table 3.

20 VII. DETAILED DESCRIPTION OF THE INVENTION

21 Applicants have discovered efficacious pharmaceutical compositions
22 wherein the relative amounts of antigen to the polymeric matrix are within the
23 ranges of 0.1 to 1.5% antigen (core loading) and 99.9 to 98.5% polymer,
24 respectively. It is preferred that the relative ratio between the lactide and

glycolide component of the poly(DL-lactide-co-glycolide) (DL-PLG) is within the range of 90:10 to 0:100. However, it is understood that effective core loads for certain antigens will be influenced by its microscopic form (i.e. bacteria, protozoa, viruses or fungi) and type of infection being prevented. From a biological perspective, the DL-PLG or glycolide monomer excipient are well suited for in vitro drug (antigen) release because they elicit a minimal inflammatory response, are biologically compatible, and degrades under physiologic conditions to products that are nontoxic and readily metabolized.

Surprisingly, applicants have discovered an extremely effective method for the protection against bacterial or viral infections in the tissue of a mammal (human or nonhuman animal) caused by enteropathogenic organisms comprising administering orally to said animal an immunogenic amount of a pharmaceutical composition consisting essentially of an antigen encapsulated within a biodegradable polymeric matrix. When the polymeric matrix is DL-PLG, the most preferred relative ratio between the lactide and glycolide component is within the range of 48:52 to 52:48. The bacterial infection can be caused by bacteria (including any derivative thereof) which include Salmonella typhi, Shigella sonnei, Shigella flexneri, Shigella dysenteriae, Shigella boydii, Escheria coli, Vibro cholera, yersinia, staphylococcus, clostridium and campylobacter. Representative viruses contemplated within the scope of this invention, susceptible to treatment with the above-described pharmaceutical compositions, are quite extensive. For purposes of illustration, a partial listing of these viruses (including any derivative thereof) include hepatitis A, hepatitis B, rotaviruses, polio virus human immunodeficiency

1 viruses (HIV), Herpes Simplex virus type 1 (cold sores), Herpes Simplex virus
2 type 2 (Herpesvirus genitalis), Varicella-zoster virus (chicken pox, shingles),
3 Epstein-Barr virus (infectious mononucleosis; glandular fever; and Burkitt's
4 lymphoma), and cytomegalo viruses.

5 A further representation description of the instant invention is as
6 follows:

7 A. (1) To homogeneously disperse antigens of enteropathic
8 organisms within the polymeric matrix of biocompatible and biodegradable
9 microspheres, 1 nanogram (ng) to 12 microns in diameter, utilizing equal
10 molar parts of polymerized lactide and glycolide (50:50 DL-PLG, i.e. 48:52 to
11 52:48 DL-PLG) such that the core load is within the range of about 0.1 to
12 1.5% by volume. The microspheres containing the dispersed antigen can then
13 be used to immunize the intestine to produce a humoral immune response
14 composed of secretory antibody, serum antibody and a cellular immune
15 response consisting of specific T-cells and B-cells. The immune response is
16 directed against the dispersed antigen and will give protective immunity against
17 the pathogenic organism from which the antigen was derived.

18 (2) AF/R1 pilus protein is an adherence factor that allows E.
19 coli RDEC-1 to attach to rabbit intestinal brush borders thus promoting
20 colonization resulting in diarrhea. AF/R1 pilus protein was homogeneously
21 dispersed within a polymeric matrix of biocompatible and biodegradable
22 microspheres, 1-12 microns in diameter (Figure 9 and photograph 1) using
23 equal molar parts of polymerized lactide and glycolide (50:50 DL-PLG) such
24 that the core load was .62% by weight.

1 (3) The microspheres were found to contain immunogenic
2 AF/R1 by immunizing both rabbit spleen (Figure 10) and Peyer's patch (Figure
3 3) B-cells in vitro. The resultant cell supernatants contained specific IgM
4 antibody which recognized the AF/R1. The antibody response was comparable
5 to immunizing with AF/R1 alone.

6 (4) Microspheres containing 50 micrograms of AF/R1 were
7 used to intrainstestinally (intraduodenally) immunize rabbits on two separate
8 occasions 1 week apart. One week later, compared to rabbits receiving AF/R1
9 alone, the intestinal lymphoid tissue, Peyer's patches, demonstrated an
10 enhanced cellular immune response to AF/R1 and to three AF/R1 linear
11 peptide fragments 40-55, 79-94 and 108-123 by both lymphocyte
12 transformation (T-cells) (Figures 12 and 13) and antibody producing B-cells
13 (Figures 14 and 15). Similarly enhanced B-cell responses were also detected in
14 the spleen (Figures 16 and 17). An enhanced T-cell response was also detected
15 in the mesenteric lymph node and the spleen to one AF/R1 peptide fragment,
16 40-55 (Figures 18 and 19). The cellular immune response at two weeks was
17 too early for either a serum or secretory antibody response (See Results in
18 Table 1); but indicates that a secretory antibody response will develop such
19 that the rabbits so immunized could be protected upon challenge with the E.
20 coli RDEC-1.

21 B. Microspheres do not have to be made up just prior to use as
22 with liposomes. Also liposomes have not been effective in rabbits for
23 intestinal immunization of lipopolysaccharide antigens.

1 C. (1) Only a small amount of antigen is required (ugs) when
2 dispersed within microspheres compared to larger amounts (mgms) when
3 antigen is used alone for intestinal immunization.

4 (2) Antigen dispersed within microspheres can be used orally
5 for intestinal immunization whereas antigen alone used orally even with gastric
6 acid neutralization requires a large amount of antigen and may not be effective
7 for intestinal immunization.

8 (3) Synthetic peptides with and without attached synthetic
9 adjuvants representing peptide fragments of protein antigens can also be
10 dispersed within microspheres for oral-intestinal immunization. Free peptides
11 would be destroyed by digestive processes at the level of the stomach and
12 intestine. Any surviving peptide would probably not be taken up by the
13 intestine and therefore be ineffective for intestinal immunization.

14 (4) Microspheres containing antigen maybe placed into
15 gelatin-like capsules for oral administration and intestinal release for improved
16 intestinal immunization.

17 (5) Microspheres promote antigen uptake from the intestine and
18 the development of cellular immune (T-cell and B-Cell) responses to antigen
19 components such as linear peptide fragments of protein antigens.

20 (6) The development of intestinal T-cell responses to antigens
21 dispersed within microspheres indicate that T-cell immunological memory will
22 be established leading to long-lived intestinal immunity. This long-lived
23 intestinal immunity (T-cell) is very difficult to establish by previous means of
24 intestinal immunization. Failure to establish long-lived intestinal immunity is a

1 fundamental difficulty for intestinal immunization with non-viable antigens.
2 Without intestinal long-lived immunity only a short lived secretory antibody
3 response is established lasting a few weeks after which no significant
4 immunological protection may remain.

5 D. (1) Oral intestinal immunization of rabbits against E. coli
6 RDEC-1 infection using either whole killed organisms, pilus protein
7 preparations or lipopolysaccharide preparations.

8 (2) Microspheres containing adherence pilus protein AF/R1 or
9 its antigen peptides for oral intestinal immunization of rabbits against RDEC-1
10 infection.

11 (3) Oral-intestinal immunization of humans against
12 enterotoxigenic E. coli infection using either whole killed organisms, pilus
13 protein preparations or lipopolysaccharide preparations.

14 (4) Microspheres containing adherence pilus proteins CFA/I,
15 II, III and IV or their antigen peptides for oral intestinal immunization of
16 humans against human enterotoxigenic E. coli infections.

17 (5) Oral-intestinal immunization of humans against other
18 enteric pathogens as salmonella, shigella, campylobacter, hepatitis-A virus,
19 rota virus and polio virus.

20 (6) Oral-intestinal immunization of animals and humans for
21 mucosal immunological protection at distal mucosal sites as the bronchial tree
22 in lungs, genito-urinary tract and breast tissue.

1 E. (1) The biocompatible, biodegradable co-polymer has a long
2 history of being safe for use in humans since it is the same one used in
3 resorbable suture material.

4 (2) By using the microspheres, we are now able to immunize
5 the intestine of animals and man with antigens not normally immunogenic for
6 the intestinal mucosa because they are either destroyed in the intestine, unable
7 to be taken up by the intestinal mucosa or only weakly immunogenic if taken
8 up.

9 (3) Establishing long-lived immunological memory in the
10 intestine is now possible because T-cells are immunized using microspheres.

11 (4) Antigens that can be dispersed into microspheres for
12 intestinal immunization include the following: proteins, glycoproteins,
13 synthetic peptides, carbohydrates, synthetic polysaccharides, lipids, glycolipids,
14 lipopolysaccharides (LPS), synthetic lipopolysaccharides and with and without
15 attached adjuvants such as synthetic muramyl dipeptide derivatives.

16 (5) The subsequent immune response can be directed to either
17 systemic (spleen and serum antibody) or local (intestine, Peyer's patch) by the
18 size of the microspheres used for the intestinal immunization. Microspheres
19 5-10 microns in diameter remain within macrophage cells at the level of the
20 Peyer's patch in the intestine and lead to a local intestinal immune response.
21 Microspheres 1 μ m - 5 microns in diameter leave the Peyer's patch contained
22 within macrophages and migrate to the mesenteric lymph node and to the
23 spleen resulting in a systemic (serum antibody) immune response.

1 (6) Local or systemic antibody mediated adverse reactions
2 because of preexisting antibody especially cytophilic or IgE antibody may be
3 minimized or eliminated by using microspheres because of their being
4 phagocytized by macrophages and the antigen is only available as being
5 attached to the cell surface and not free. Only the free antigen could become
6 attached to specific IgE antibody bound to the surface of mast cells resulting in
7 mast cell release of bioactive amines necessary for either local or systemic
8 anaphylaxis.

9 (7) Immunization with microspheres containing antigen leads
10 to primarily IgA and IgG antibody responses rather than an IgE antibody
11 response, thus preventing subsequent adverse IgE antibody reactions upon
12 reexposure to the antigen.

13 In addition to the above, the encapsulation of the following synthetic
14 peptides are contemplated and considered to be well within the scope of this
15 invention:

- 16 (1) AF/R1 40-55;
- 17 (2) AF/R1 79-94;
- 18 (3) AF/R1 108-123;
- 19 (4) AF/R1 1-13;
- 20 (5) AF/R1 pepscan 16AA;
- 21 (6) CFA/I 1-13; and
- 22 (7) CFA/I pepscan 16AA.
- 23 (8) Synthetic Peptides Containing CFA/I Pilus Protein
- 24 T-cell Epitopes (Starting Sequence # given)

1 4(Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro),
2 8(Thr-Ala-Ser-Val-Asp-Pro-Val-Ile-Asp-Leu),
3 12(Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
4 15(Ile-Asp-Leu-Leu-Gln-Ala-Asp-Gly-Asn-Ala),
5 20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),
6 26(Pro-Ser-Ala-Val-Lys-Leu-Ala-Tyr-Ser-Pro),
7 72(Leu-Asn-Ser-Thr-Val-Gln-Met-Pro-Ile-Ser),
8 78(Met-Pro-Ile-Ser-Val-Ser-Trp-Gly-Gly-Gln),
9 87(Gln-Val-Leu-Ser-Thr-Thr-Ala-Lys-Glu-Phe),
10 126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr), and
11 133(Gly-Asn-Tyr-Ser-Gly-Val-Val-Ser-Leu-Val), and mixtures
12 thereof.

13 (9) Synthetic Peptides Containing CFA/I Pilus Protein B-cell (antibody)

14 Eptiopes (Starting Sequence # given)

15 3(Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val),
16 11(Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
17 22(Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),
18 32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-
19 Glu-Ser-Tyr-Arg-Val),
20 32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe),
21 38(Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),
22 66(Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser),
23 93(Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala),
24 124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr),

1 127(Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and
2 124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-
3 Ser), and mixtures thereof.

4 (10) synthetic peptides containing CFA/I pilus protein T-cell and
5 B-cell (antibody) epitopes (Starting Sequence # given)

6 3(Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Bal-Asp-Pro),
7 8(Thr-Ala-Ser-Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
8 11(Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
9 20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),
10 124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and
11 126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and mixtures
12 thereof.

13 (11) synthetic peptides containing CFA/I pilus protein T-cell and
14 B-cell (antibody) epitopes (Starting Sequence # given)

15 CFA/I pilus protein T-cell epitopes

16 4(Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro),
17 8(Thr-Ala-Ser-Val-Asp-Pro-Val-Ile-Asp-Leu),
18 12(Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
19 15(Ile-Asp-Leu-Leu-Gln-Ala-Asp-Gly-Asn-Ala),
20 20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),
21 26(Pro-Ser-Ala-Val-Lys-Leu-Ala-Tyr-Ser-Pro),

1 72(Leu-Asn-Ser-Thr-Val-Gln-Met-Pro-Ile-Ser),
2 78(Met-Pro-Ile-Ser-Val-Ser-Trp-Gly-Gly-Gln),
3 87(Gln-Val-Leu-Ser-Thr-Thr-Ala-Lys-Glu-Phe),
4 126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr), and
5 133(Gly-Asn-Tyr-Ser-Gly-Val-Val-Ser-Leu-Val); and synthetic
6 peptides containing CFA/I pilus protein B-cell (antibody) epitopes (Starting
7 Sequence # given)

8 CFA/I pilus protein B-cell epitopes
9 3(Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val),
10 11(Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
11 22(Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),
12 32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-
13 Glu-Ser-Tyr-Arg-Val),
14 32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe),
15 38(Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),
16 66(Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser),
17 93(Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala),
18 124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr),
19 127(Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and
20 124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-
21 Ser), and mixtures thereof.

(12) synthetic peptides containing CFA/I pilus protein T-cell and B-cell (antibody) epitopes (Starting Sequence # given)

CFA/I pilus protein T-cell epitopes

3(Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val),

11(Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

22(Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),

32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe),

38(Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),

66(Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser),

93(Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala),

124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr),

127(Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and

124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-

Ser); and

synthetic peptides containing CFA/I pilus protein T-cell and B-cell (antibody) epitopes (Starting Sequence # given)

CFA/I pilus protein B-cell epitopes

3(Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Bal-Asp-Pro),

8(Thr-Ala-Ser-Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

1 11(Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
2 20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),
3 124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and
4 126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and mixtures
5 thereof.

6 We contemplate that the peptides can be used in vaccine constructed
7 for systemic administration.

8 EXAMPLES

9 The peptides in (8), (9), and (10) above can be made by classical
10 solution phase synthesis, solid phase synthesis or recombinant DNA
11 technology. These peptides can be incorporated in an oral vaccine to prevent
12 infection by CFA/I bearing enteropathogenic E. coli.

13 The herein offered examples provide methods for illustrating, without
14 any implied limitation, the practice of this invention in the prevention of
15 diseases caused by enteropathogenic organisms.

16 The profile of the representative experiments have been chosen to
17 illustrate the effectiveness of the immunogenic polymeric matrix-antigen
18 composites.

19 All temperatures not otherwise indicated are in degrees Celcius (°C)
20 and parts or percentages are given by weight.

21 MATERIALS AND METHODS

22 Animals. New Zealand White male rabbits were purchased from
23 Hazelton Research Products (Denver, PA), and were shown to be free of
24 current RDEC-1 infection by culture of rectal swabs. Animals were 1-2 kg of

1 body weight and lacked agglutinating anti-AF/R1 serum antibody at the time of
2 the study.

3 Antigens. AF/R1 pili from E. coli RDEC-1 (015:H:K non-typable)
4 were purified by an ammonium sulfate precipitation method. The final
5 preparation migrated as a single band on SDS-polyacrylamide gel
6 electrophoresis and was shown to be greater than 95% pure by scanning with
7 laser densitometry when stained with coomassie blue. Briefly, equal molar
8 parts of DL-lactide and glycolide were polymerized and then dissolved to
9 incorporate AF/R1 into spherical particles. The microspheres contained 0.62%
10 protein by weight and ranged in size from 1 to 12 micrometers. Both the
11 microencapsulated and non-encapsulated AF/R1 were sterilized by gamma
12 irradiation (0.3 megarads) before use.

13 Synthetic peptides (16 amino acids each) were selected by theoretical
14 criteria from the amino acid sequence of AF/R1 as deduced from the
15 nucleotide sequence. Three sets of software were used for the selections.
16 Software designed to predict B cell epitopes based on hydrophilicity,
17 flexibility, and other criteria was developed by the University of Wisconsin
18 Genetics Computer Group. Software designed to predict T cell epitopes was
19 based on the Rothbard method was written by Stephen Van Albert (The Walter
20 Reed Army Institute of Research, Washington, D.C.). Software designed to
21 predict T cell epitopes based on the Berzofsky method is published as the
22 AMPHI program. The selected peptides were synthesized by using
23 conventional Merrifield solid phase technology. AF/R1 40-55
24 (Thr-Asn-Ala-Cly-Thr-Asp-Ile-Gly- Ala-Asn-Lys-Ser-Phe-Thr-Leu-Lys) was

1 various dilutions of antigen and were incubated at 37°C in 5% CO₂. In other
2 experiments, cultures were conducted in 24-well plates. In these
3 experiments, 5 x 10⁶ cells were cultured with or without antigen in a 2 ml
4 volume. After 4 days, 100 microliters aliquots of cells were transferred to
5 96-well plates for pulsing and harvesting. Previous experiments have
6 demonstrated that optimal concentrations of antigen range from 150 ng/ml to
7 15 micrograms/ml in the 96-well plate assay and 1.5 ng/ml to 150 ng/ml in the
8 24-well plate assay. These were the concentrations employed in the current
9 study. All cultures were pulsed with 1 Ci [³H]thymidine (25 Ci/mmol,
10 Amersham, Arlington Heights, IL) on day 4 of culture and were harvested for
11 scintillation counting 6 hours later.

12 Statistics. All cultures were conducted in replicates of four, and
13 standard deviations of the counts per minute (cpm) generally range from
14 5-15% of the average cpm. In experiments where comparison of individual
15 animals and groups of animals is desirable, data is shown as a stimulation
16 index (SI) to facilitate the comparison. SI were calculated by dividing the mean
17 of cultures with antigen by the mean of cultures without antigen (media
18 control). Statistical significance (p value) was determined by comparing the
19 maximum response for each antigen to the media control using the Student's t
20 test.

21 RESULTS

22 Lymphocyte proliferation in response to protein and peptide antigens
23 of AF/R1. To determine if lymphoid tissues from AF/R1 immune animals
24 respond in vitro to the antigens of AF/R1, the immunity in a rabbit with

1 preexisting high levels of anti-AF/R1 serum IgG was boosted twice by
2 injection of 50 micrograms of purified AF/R1 pili i.p. seven days apart. A
3 week after the final boost, *in vitro* lymphocyte proliferation of spleen and
4 MLN cells demonstrated a remarkable response to AF/R1 pili. In
5 response to the synthetic peptides, there was a small, but significant
6 proliferation of the spleen cells to all the AF/R1 peptides tested as compared to
7 cell cultures without antigen. Cells from the spleen and Peyer's
8 patches of non-immune animals failed to respond to either AF/R1 or the
9 synthetic peptides.

10 Microencapsulation of AF/R1 potentiates the mucosal cellular immune
11 response. To evaluate the effect that microencapsulation of AF/R1 may have
12 on the cellular mucosal immune response to that antigen, naive rabbits were
13 primed twice with 50 micrograms of either microencapsulated or
14 non-encapsulated AF/R1 by endoscopic intraduodenal inoculation seven days
15 apart. All rabbits were monitored daily and showed no evidence of clinical
16 illness or colonization by RDEC-1. One week following the last priming, the
17 rabbits were sacrificed and lymphoid tissues were cultured in the presence of
18 AF/R1 pili or peptide antigens. In rabbits which had received
19 non-encapsulated AF/R1, Peyer's Patch cells demonstrated a low level but
20 significant proliferation *in vitro* in response to AF/R1 pili (Fig 13), but not to
21 any of the AF/R1 synthetic peptides (Fig 14a-d). However, in rabbits which
22 had received microencapsulated AF/R1, Peyer's Patch cells demonstrated a
23 markedly enhanced response not only to AF/R1 (Fig 13) but now responded to
24 the AF/R1 synthetic peptides 40-55 and 79-94 (Fig 14a and 14b). In addition,

1 one of two rabbits primed with microencapsulated AF/R1 (rabbit 135)
2 responded to AF/R1 108-123, but not AF/R1 40-47/79-86 (Fig 4c and 4d).
3 In contrast, the other rabbit in the group (rabbit 134) responded to AF/R1
4 40-47/79-86, but not to AF/R1 108-123 (Fig 4d and 4c).

5 Response of MLN cells to the antigens of AF/R1. Studies have shown
6 that cells undergoing blastogenesis in the MLN also tend to home into mucosal
7 areas, but experiments requiring *in vitro* lymphocyte proliferation of rabbit
8 MLN cells are difficult to conduct and to interpret due to non-specific high
9 background cpm in the media controls. Our studies have shown that this
10 problem can be avoided by conducting the proliferative studies in 24-well
11 plates, and then moving aliquots of cells into 96-well plates for pulsing with
12 [³H]thymidine as described in materials and methods. This method of culture
13 was employed for the remainder of the studies. The MLN cells of all rabbits
14 demonstrated a significant proliferation *in vitro* in response to AF/R1 pili
15 regardless of whether they had been immunized with microencapsulated or
16 non-encapsulated AF/R1. However, only the rabbits which had
17 received microencapsulated AF/R1 were able to respond to the AF/R1
18 synthetic peptide 40-55 (Fig. 19). The MLN cells of rabbit 134 also
19 responded to AF/R1 79-94 ($p < 0.0001$), AF/R1 108-123 ($p < 0.0001$), and
20 AF/R1 40-47/79-86 ($p = 0.0004$); however, none of the other rabbits
21 demonstrated a MLN response to those three peptides (data not shown).

22 Response of spleen cells to the antigens of AF/R1. Proliferative
23 responses of spleen cells to AF/R1 were very weak in all animals tested (data
24 not shown). However, in results which paralleled the responses in MLN cells,

1 there was a significant response to AF/R1 40-55 in rabbits which had been
2 primed with microencapsulated AF/R1 (Fig. 20'). There was no response to
3 the other AF/R1 synthetic peptides by spleen cells in either group of animals.
4 The weak response of spleen cells to AF/R1 provides further evidence that
5 these animals were naive to AF/R1 before the study began, and indicates that
6 the observed responses were not due to non-specific stimulative factors such as
7 lipopolysaccharide.

8 SUMMARY

9 We have shown that there is an enhanced in vitro proliferative
10 response to both protein and its peptide antigens by rabbit Peyer's patch cells
11 following intraduodenal inoculation of antigen which had been homogeneously
12 dispersed into the polymeric matrix of biodegradable, biocompatible
13 microspheres. The immunopotentiating effect of encapsulating purified AF/R1
14 pili as a mucosal delivery system may be explained by one or more of the
15 following mechanisms: (a) Microencapsulation may help to protect the antigen
16 from degradation by digestive enzymes in the intestinal lumen. (b)
17 Microencapsulation has been found to effectively enhance the delivery of a
18 high concentration of antigen specifically into the Peyer's patch. (c) Once
19 inside the Peyer's patch, microencapsulation appears to facilitate the rapid
20 phagocytosis of the antigen by macrophages, and the microspheres which are
21 5-10 micrometers become localized within the Peyer's patch. (d)
22 Microencapsulation of the antigen may improve the efficiency of antigen pre-
23 sentation by decreasing the amount of enzymatic degradation that takes place
24 inside the macrophage before the epitopes are protected by combining with

1 Class II major histocompatibility complex (MHC) molecules. (e) The slow,
2 controlled-release of antigen may produce a depot effect that mimics the
3 retention of antigen by the follicular dendritic cell. (f) If the antigen of interest
4 is soluble, microencapsulation changes the antigen into a particulate form
5 which appears to assist in producing an IgA B cell response by shifting the
6 cellular immune response towards the T_H and thereby not encouraging a
7 response by the T_H . There is evidence that the GALT may be able to
8 discriminate between microbial and non-microbial (food) antigens in part by
9 the form of the antigen when it is first encountered, and thus bacterial antigens
10 do not necessarily have special antigenic characteristics that make them
11 different from food antigens, but they are antigenic because of the bacterial
12 context in which they are presented. The particulate nature of microspheres
13 may serve to mimic that context. It may be important to note that we also
14 observed a significant response to AF/R1 in animals inoculated with
15 non-encapsulated pili; thus, some of this antigen which was still in its native
16 form was able to enter the Peyer's patch. This may be explained by the fact
17 that AF/R1 is known to mediate the attachment of RDEC-1 to the Peyer's
18 patch M-cell. If the antigen employed in this type of study was not able to
19 attach to micrometer M-cells, one would expect to see an even greater
20 difference in the responses of animals which had received microencapsulated
21 versus non-encapsulated antigen.

22 The microspheres used in these experiments included a size range
23 from 1 to 12 micrometers. The 1 to 5 micrometer particles have been shown
24 to disseminate to the MLN and spleen within migrating macrophages; thus, the

1 observed proliferative responses by cells from the MLN and spleen may reflect
2 priming of MLN or splenic lymphocytes by antigen-presenting/accessory cells
3 which have phagocytosed 1 to 5 micrometer antigen-laden microspheres in the
4 Peyer's patch and then disseminated onto the MLN. Alternatively, these
5 responses may be a result of the normal migration of antigen stimulated
6 lymphocytes that occurs from the Peyer's patch to the MLN and on into the
7 general circulation before homing to mucosal sites. Proliferative responses by
8 MLN cells are of interest because it has been shown that cells undergoing
9 blastogenesis in the MLN tend to migrate onto mucosal areas. However,
10 studies involving in vitro lymphocyte proliferation of rabbit MLN cells can be
11 very difficult to conduct and to interpret due to non-specific high background
12 cpm in the media controls. By simultaneously conducting experiments using
13 different protocols, we have found that this problem can be prevented by
14 avoiding the use of fetal calf serum in the culture and by initially plating the
15 cells in 24-well plates. Using this method, the blasting lymphocytes are easily
16 transferred to a 96-well plate where they receive the [³H]thymidine, while
17 fibroblasts and other adherent cells remain behind and thus do not inflate the
18 background cpm.

19 The proliferative response to the peptide antigens was of particular
20 interest in these studies. The rabbits that received non-encapsulated AF/R1
21 failed to respond to any of the peptides tested either at the level of the Peyer's
22 patch, the MLN, or the spleen. In contrast, Peyer's patch cells from the
23 animals that received microencapsulated AF/R1 responded to all the peptides
24 tested with two exceptions: Rabbit 134 did not respond to AF/R1 108-123,

1 and rabbit 135 did not respond to AF/R1 40-47/79-86. The reason for these
2 non-responses is not clear, but it probably is not due to MHC restrictions as
3 evidenced by the fact that rabbit 134 was able to respond to AF/R1 108-123 at
4 the level of the MLN. The non-responses may be due to varying kinetics of
5 sensitized T cell migration in different rabbits, or they may reflect differences
6 in the efficiency of antigen presentation by cells from different lymphoid
7 tissues of these animals. Of all the synthetic peptides tested, only AF/R1
8 40-55, (the one selected as a probable B cell epitope), was recognized by
9 serum from an AF/R1 hyperimmune rabbit. In addition, this peptide was the
10 only one that was uniformly recognized by Peyer's patch, MLN, and spleen
11 cells from both rabbit. In addition, this peptide was the only one that was
12 uniformly recognized by Peyer's patch, MLN, and spleen cells from both
13 rabbits that were immunized with microencapsulated AF/R1. The recognition
14 by anti-AF/R1 serum antibodies indicates that the amino acid sequence of this
15 peptide includes an immunodominant B cell epitope. Thus AF/R1 40-55 may
16 readily bind to antigen-specific B cells thereby leading to an efficient B cell
17 presentation of this antigen to sensitized T cells. Even though AF/R1 40-55
18 was not selected as a probable T cell epitope by either the Rothbard or
19 Berzofsky methods, the current study clearly indicates that this peptide can also
20 stimulate a proliferative immune response. Although further studies are
21 required to definitively show that the proliferating cells are indeed T cells, the
22 responses observed in this study are most likely due to the blast transformation
23 of cells from the lineage. Therefore, AF/R1 40-55 appears to contain a T cell
24 epitope in addition to the immunodominant B cell epitope, and this area of the

1 AF/R1 protein may thereby play an important role in the overall immune
2 response and subsequent protection against RDEC-1.

3 The proliferative responses of spleen cells was low in all animals
4 tested; however, we feel tht this may be simply a matter of the kinetics of
5 cellular migration. The rabbits in this study were sacrificed only two weeks
6 after their first exposure to antigen. This relatively short time period may not
7 have provided sufficient time for cells that were produced by Peyer's patch and
8 MLN blasts to have migrated as far as the spleen in sufficient numbers.

9 An ideal mucosal vaccine preparation would not only assist in the
10 uptake and presentation of the immunogen of interst, but it would also be
11 effective without requiring carrier molecules or adjuvants which may
12 complicate vaccine production or delay regulatory approval. The incorporation
13 of antigen into microspheres appears to provide an ideal mucosal delivery
14 system for oral vaccine immunogens because the observed immunopotentiating
15 effect is achieved without the need for carriers of adjuvants. This ability may
16 prove to be of great value, particularly to enhance the delivery of oral
17 synthetic peptide vaccines to the GALT.

18 TABLE 10 Linear B-Cell Epitopes of CFA/I in Monkeys

19	Sequence		Individuals
20	<u>Position</u>	<u>Responding</u>	<u>Consensus Site</u>
21	1. 11-21	3	VDPVIDLLQ

1	2. 93-101	2	AKEFEAAA
2	3. 124-136	2	GPAPT
3	4. 66-74	2	PQLTDVLN
4	5. 22-29	2	GNALPSAV
5	6. 32-40	1	KTF*
6	7. 38-45	1	
7	8. 3-11	1	

8

9 *Overlap between epitope 6 and 7

TABLE 11

Prediction of T cell epitopes within the CFA/I molecule*

<u>Predicted Amphipathic Segments</u>		<u>Rothbard Criteria</u>
7 aa blocks	11 aa blocks	
22-25	8-11	16
34-38	32-44	30
40-46	51-71	38
50-53	86-92	44
56-62	102-108	57
64-71	130-131	61
104-108	135-137	70
131-137		116
		124
		127
		137

*The sequence numbers of the first amino acid of the predicted T cell epitopes are shown. Software designed to predict T cell epitopes based on the Berzofsky method was published as the AMPHI program. It predicts amphipathic amino acid segments by evaluating 7 or 11 residues as a block and assigning a score to the middle residue of that block. Software designed to predict T cell epitopes based on the Rothbard method was written by Stephen Van Albert (The Walter Reed Army Institute of Research, Washington, D.C.).

TABLE 11

Amino acid sequence of immunodominant T cell epitopes*

Residue	
Numbers	Amino Acids
8-17	Thr Ala Ser Val Asp Pro Val Ile Asp Leu
40-49	Phe Glu Ser Tyr Arg Val Met Thr Gln Val
72-81	Leu Asn Ser Thr Val Gln Met Pro Ile Ser
134-143	Asn Tyr Ser Gly Val Val Ser Leu Val Met

*Of the 19 decapeptides that supported a significant proliferative response and contained a serine at either position 2, 3, or 4, nine has a serine specifically at position 3. Some of the most robust responses were to the peptides that contained a serine residue at the third position. The amino acid sequence of four such decapeptides which are believed to be immunodominant T cell epitopes is shown.

DEMONSTRATIVE EVIDENCE OF PROTECTIVE IMMUNITY

RDEC-1 is an enteroadherent diarrhea producing E. coli in rabbit. Its attachment to the mucosa is by the adhesin (AF/R1 pili). The adhesin is an excellent vaccine candidate. It may initiate a mucosal response but is susceptible to digestion in the gut. The incorporation of AF/R1 into biocompatible, nondigestible microspheres enhanced mucosal cellular immune responses to RDEC-1. We have demonstrated that immunization with AF/R1 Pili in microspheres protect rabbits against infection with RDEC-1.

Six rabbits received intra-duodenal immunization of AF/R1 microspheres (0.62% coreloading by weight) at 200 ug AF/R1 on day 0 then boosted with 100 ug AF/R1 in microspheres on days 7, 14, and 21 followed

1 by RDEC-1 challenge with 10^8 organisms one week latter than bserved for 1
2 week and then sacrificed, unimmunized rabbits were challenged with 10^8
3 RDEC-1 only and observed 1 week than sacrificed. Also, 2 rabbits were
4 immunized only then were sacrificed 10 days latter. Only one of these animals
5 had bile IgA antibodies to AF/R1. but both had specific sensitized T cells
6 which released IL-4 upon challenge in the spleen, Peyer's patch and illeal
7 lamina propria. All nine immunized animals developed diarrhea and weight
8 loss which was significant at the $p < .001$ level compared to the immunized
9 animals which displayed no diarrhea and no weight loss. The immunized
10 animals colonized the intestinal tract with RDEC-1 the same as the
11 unimmunized animals. However, there was a striking difference regarding the
12 adherence of RDEC-1 to the mucosa. No adherence was seen in cecum in the
13 immunized animals compared to 4/7 in the unimmunized side animals. This
14 difference was significant to the $p < .01$ level. The RDEC-1 exposure
15 although not producing disease in the immunized animals did effect a booster
16 immunization as relected in the increase in anti-AF/R1 antibody containing
17 cells in the muscosa similiar to the immunized rabbits. This study clearly
18 demonstrated complete protection against RDEC-1 infection and strongly
19 indicates similiar results should be expected with enterotoxigenicity E. coli
20 using the Colony Forming Antigens (CFA's) in microsphere vaccines.

21 SUMMARY STATEMENT OF PROTECTIVE IMMUNITY SHOWINGS

22 RDEC-1 infection of rabbits causes an enteroadherent E. coli diarrheal
23 disease, and provides a model for the study of adherence-factor immunity.
24 Pilus adhesions are vaccine candidates, but purified pili are subject to intestinal

1 degradation. Previously we showed potentiation of the mucosal cellular
2 immune response to the AF/R1 pilus of RDEC-1 by incorporation into
3 biodegradable polylactide-coglycolide microspheres (AF/R1-MS). We now
4 present efficacy testing of this vaccine. Six rabbits were primed with 200 ug
5 and boosted with 100 ug of AF/R1-MS weekly x3, then challenged at week 5
6 with 10^8 CFU of RDEC-1 expressing AF/R1. Nine unvaccinated rabbits were
7 also challenged. Two rabbits vaccinated with AF/R1-MS were sacrificed at
8 week 5, without challenge, for measurement of anti-AF/R1 antibodies in bile
9 (by ELISA) and anti-AF/R1 containing cells (ACC) in the intestinal lamina
10 propria (by immunohistochemistry). Attachment of RDEC-1 to intestinal
11 epithelial cells was estimated (0.4+) by immunoperoxidase staining of
12 histologic sections. Colonization of intestinal fluid was measured by culture of
13 intestinal flushes. Results: Rabbits given AF/R1-MS remained well and 4/6
14 gained weight after challenge, whereas 9/9 unvaccinated rabbits lost weight
15 after challenge (mean weight change +10 vs -270 gms $p < .001$), (see Figure
16 35). The mean score of RDEC-1 attachment to the cecal epithelium was 0 in
17 vaccinated, and 2+ in unvaccinated animals (see Figure 36). RDEC-1
18 colonization (log CFU/gm) in cecal fluids was similar in both groups (mean
19 6.3 vs 7.3; $p = .09$) (see Figure 34). ACC were not seen in the lamina propria
20 of vaccinated but unchallenged animals, but anti-pilus IgA antibody levels in
21 bile were increased 1 S.D. over negative controls in 1 animal. Conclusions:
22 Vaccination with AF/R1-MS was safe and protected rabbits against RDEC-1
23 disease. Protection was associated with interference with RDEC-1 adherence
24 to the mucosal surface, but luminal colonization was not prevented.

101

More recent applicants have focused areas of this invention related to an immunostimulating composition for the burst-free, sustained, programmable release of active material(s) over a period from 1 to 100 days, which comprises encapsulating microspheres, which may contain a pharmaceutically-acceptable adjuvant, wherein said microspheres are comprised of (a) a blend of uncapped and end-capped biodegradable-biocompatible poly(DL-lactide-co-glycolide) as the bulk matrix, wherein the relative ratio between the amount of lactide and glycolide components are within the range of 90:10 to 40:60 and the poly(DL-lactide-co-glycolide) is a blend of uncapped and end-capped forms in ratios ranging from 100:0 to 1 to 99, and (b) active material such as an immunogenic substance comprising Colony Factor Antigen (CFA/II, hepatitis B surface antigen (HBsAg)), and/or a physiologically similar antigen that serves to elicit the production of antibodies in a mammal (human or nonhuman).

These areas of invention are referred to herein after as Part II and Part III, respectively, and are itemized as follows:

1. An immunostimulating composition for the burst-free, sustained, programmable release of active material(s) over a period from 1 to 100 days, which comprises encapsulating-microspheres, which may contain a pharmaceutically-acceptable adjuvant, wherein said microspheres having a diameter between 1 nanogram (ng) to 10 microns (um) are comprised of (a) a blend of uncapped and end-capped biodegradable-biocompatible poly (DL-lactide-co-glycolide) as the bulk matrix, wherein the relative ratio between the amount of lactide and glycolide components are within the range of 90:10 to 40:60, and the poly(DL-lactide-co-glycolide) is a blend of uncapped and end-capped forms in ratios ranging from 100:0 to 1 to 99, and (b) active material such as an immunogenic substance comprising Colony Factor Antigen (CFA/II), hepatitis B surface antigen (HBsAg), and/or a physiologically similar antigen that serves to elicit the production of antibodies in a mammal (human or nonhuman).

- 1 2. An immunostimulating composition according to Item 1 wherein the
2 amount of said immunogenic substance is within the range of 0.1 to 1.5%
3 based on the volume of said bulk matrix.
- 4 3. An immunostimulating composition according to Item 2 wherein the
5 relative ratio between the lactide and glycolide component is within the range
6 of 48:52 to 52:48.
- 7 4. An immunostimulating composition according to Item 2 wherein the size
8 of more than 50% of said microspheres is between 5 to 10 μ m in diameter by
9 volume.
- 10 5. A vaccine comprising an immunostimulating composition of Item 4 and a
11 sterile, pharmaceutically-acceptable carrier therefor.
- 12 6. A vaccine comprising an immunostimulating composition of Item 5
13 wherein said immunogenic substance is Colony Factor Antigen (CFA/II).
- 14 7. A vaccine comprising an immunostimulating composition of Item 5
15 wherein said immunogenic substance is hepatitis B surface antigen (HBsAg).
- 16 8. A method for the vaccination against bacterial infection comprising
17 administering to a human, an antibactericidally effective amount of a
18 composition of Item 6.

1 9. A method according to Item 7 wherein the bacterial infection is caused by
2 a bacteria selected from the group consisting essentially of Salmonella typhi,
3 Shigella Sonnei, Shigella Flexneri, Shigella dysenteriae, Shigella boydii,
4 Escheria coli, Vibrio cholera, yersinia, staphylococcus, clostridium, and
5 campylobacter.

6 10. A method for the vaccination against viral infection comprising
7 administering to a human an antivirally effective amount of a composition of
8 Item 7.

9 11. A diagnostic assay for bacterial infections comprising a composition of
10 Item 4.

11 12. A method of preparing an immunotherapeutic agent against infections
12 caused by a bacteria comprising the step of immunizing a plasma donor with a
13 vaccine according to Item 6 such that a hyperimmune globulin is produced
14 which contains antibodies directed against the bacteria.

15 13. A method preparing an immunotherapeutic agent against infections caused
16 by a virus comprising the step of immunizing a plasma donor with a vaccine
17 according to Item 7 such that hyperimmune globulin is produced which
18 contains antibodies directed against the hepatitis B virus.

1 14. An immunotherapy method comprising the step of administering to a
2 subject an immunostimulatory amount of hyperimmune globulin prepared
3 according to Item 12.

4 15. An immunotherapy method comprising the step of administering to a
5 subject an immunostimulatory amount of hyperimmune globulin prepared
6 according to Item 13.

7 16. A method for the protection against infection of a mammal (human or
8 nonhuman animal) by enteropathogenic organisms or hepatitis B virus
9 comprising administering to said mammal an immunogenic amount of an
10 immunostimulating composition of Item 3.

11 17. A method according to Item 16 wherein the immunostimulating
12 composition is administered orally.

13 18. A method according to Item 16 wherein the immunostimulating
14 composition is administered parenterally.

15 PART II

16 In sum, the Colony Factor Antigen (CFA/II) from enterotoxigenic E
17 coli (ETEC) prepared under GMP was successfully incorporated into
18 biodegradable polymer microspheres (CFA/II BPM) under GMP and found to
19 be safe and immunogenic when administered intra-duodenally to rabbits.
20 CFA/II was incorporated into poly (D,L-lactide-co-glycolide) (PLGA)

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1 The first step in pathogenesis is adherence to the small intestine
2 epithelial cells by protein fimbrial (pilus) adhesins called colonization factor
3 antigen (CFA). Three major CFAs have been recognized, CFA/I, CFA/II and
4 CFA/IV. (25)

5 Ten human volunteers who were immunized orally twice weekly for 4
6 weeks with CFA/II developed a poor antibody response and did not show any
7 significant protection when challenged with pathogenic ETEC (26). This
8 disappointing response was attributed to adverse effects of gastric acid, even at
9 neutral pH, of fimbrial proteins (27). When the vaccine was administered by
10 inoculation directly into the duodenum, 4 of 5 immunized volunteers developed
11 a significant rise in secretory IgA with CFA/II antibody (26).

12 D and L-lactic acid and glycolic acid, as homo- and
13 copolymers, are biodegradable and permit slow and continued release
14 of antigen with a resultant adjuvant activity. These polymers have
15 been shown to be safe in a variety of applications in human beings
16 and in animals (28-32). Delivery of antigens via microspheres
17 composed of biodegradable, biocompatible lactide/glycolide
18 polymers (29-32) may enhance the mucosal response by protecting the
19 antigen from digestion and targeting them to lymphoid cells in
20 Peyer's patches (29-32). McQueen et al. (33) have shown that E coli AF/R1
21 pili in PLGA microspheres, introduced intra-duodenally in rabbits, protected
22 them against diarrhea and weight loss when challenged with the parent strain

1 rabbit diarrheagenic strain of E coli (RDEC-1). Only one vaccinated rabbit of
2 six lost weight and only one had soft pelleted stool. In contrast, all control
3 unvaccinated animals became ill, lost weight, and shed soft pellets
4 or unformed mucoid stool. Significant lymphocyte proliferation to
5 AF/R1 from Peyer's patches and ordinary IgA anti AF/R1 antibody
6 levels were seen.

7 In order to improve the CFA/II vaccine it was incorporated
8 into PLGA microspheres under GMP in order to protect it from
9 digestion and target it to the intestinal lymphoid system. The
10 CFA/II BPM vaccine has undergone pre-clinical evaluation and has
11 been found to be safe and immunogenic.

12 MATERIALS AND METHODS

13 Preparation of CFA/II Pilus Vaccine. Under Good Laboratory and
14 Good Manufacturing Practices, E. coli, strain M424C1-06;816 producing
15 CFA/II were cultured in 75-80 CFA agar plates (24 x 24 cm) for 24 hrs then
16 harvested by scraping. The harvest was homogenized at slow speed for 30
17 minutes with over head drive unit and cup immersed in an ice bath. The
18 homogenate was centrifuge at 4° C at 16, 500 x g for 30 minutes. The
19 supernatant saved and the pellet rehomogenized and centrifuged with the
20 supernatants pooled. The supernatant pool was centrifuged at 50,000 x g for
21 45 minutes. The supernatant treated with ammonium sulfate at 20%
22 saturation, stirred 30 minutes at 4° C than stored at 4° C for 16 hrs then

1 centrifuged at 19,700 x g for 30 minutes. The supernatant saved and treated
2 with ammonium sulfate at 45% saturation, stirred 30 minutes at 4° C, stored at
3 4° C for 66-72 hrs, then centrifuged at 19,700 x g for 45 minutes. The pellet
4 was resuspended in about 100 mls of PBS containing 0.5% formalin and held
5 at 22° for 18 hrs then dialyzed for 45-50 hrs against PBS at 4° C using a total
6 of 12 liters in 2 liter amounts. The dialysis was terminated when the PBS
7 contained less than 0.03% formalin using Nessler's reagent and fuchsin
8 sulfuose acid reagent. The final product contained 1 mgm protein/ml PBS,
9 was sterile and passed the general safety test.

10 Preparation of Desalted CFA/II Vaccine. Two ml of the CFA/II
11 vaccine were placed into a Centricon 30 tube and centrifuged at 1700 rpm at 4-
12 6° C (Beckman model GPR centrifuge equipped with GA-24 fixed angle rotor)
13 until all the buffer solution passed through the filter (about 90-120 minutes).
14 Sterile water was added to each tube to disperse the CFA/II retained on the
15 filter. The desalted antigen dispersions from all tube were pooled and then
16 divided into five equal parts by weight so as to contain 20 mg of the CFA/II
17 each. The desalted antigen dispersion was stored at -10 to -20° C.

18 Freeze Drying of the Desalted CFA/II Dispersion. 80 mg of sucrose
19 was added to each part of the CFA/II dispersion. The resulting mixture was
20 flash-frozen using a dry ice-acetone bath (100-150 ml of acetone and 50-100 g
21 of dry ice). The frozen solution was freeze dried overnight using Repp
22 Sublimator 16 freeze dryer at vacuum of 1 micrometer of mercury and a shelf
23 temperature not exceeding 37° C.

CFA/II Biodegradable Polymer Microspheres.

Particle size distribution. About 1 mgm of microspheres were dispersed in 2 ml of 1% Polysorbate 60[®] (Ruger Chemical Co. Inc. Irvington, N.J.) in water in a 5 ml capacity glass vial by sonication. This dispersion was observed under a calibrated optical microscope with 43x magnification. Using a precalibrated eye-piece micrometer, the diameter of 150 randomly chosen microspheres, was determined and the microsphere size distribution was determined

Scanning Electron Microscopic Analysis. Microspheres were sprinkled on the surface of 10mm stub covered with a non-conductive adhesive (Sticky-Tab, Ernest F. Fullem, Inc., Lutham, N.Y.) Samples were coated with gold/palladium in an automatic sputter-coating apparatus (Samsputter-2A, Tonsimis Research Corporation). The samples were examined with a Hitachi S-450 scanning electron microscope operated at 15-20 KV.

Preparation of CFA/II Microspheres. Solvent extraction technique was used to encapsulate the freeze dried CFA/II into poly(lactide-co-glycolide)(Medisorb Technologies International, viscosity 0.73 dl/g) microspheres in the 1-10 um size range to achieve theoretical antigen loading of 1% by weight. The freeze dried antigen-sugar & matrix was dispersed in an acetonitrile solution of the polymer and then emulsified to achieve desired droplet size. Microspheres were solidified and recovered by using heptane as extracting solvent. The microsphere batches were pooled and vacuum dried to remove traces of solvent.

1 Protein Content. The CFA/II microspheres were dissolved in 0.9%
2 SDS in 0.1N NaOH for 18 hr with stirring then neutralized to pH 7
3 and assayed. The micro bicichoninic acid (BCA) method was utilized with
4 both lactic acid and glycolic acid blanks and compared to
5 bovine serum albumin (BSA) standard and results expressed as percent by
6 weight.

7 Moisture Content. One hundred and fifty mgm of CFA/II
8 microspheres were dissolved in 3 ml of acetonitrile by sonication for 3 hours.
9 One ml sample was injected into a Karl Ficher titrimeter and triter reading
10 observed was recorded and acetonitrile blank was subtracted to determined
11 percent water content.

12 Acetonitrile and Heptane Residuals. Ten mgm of CFA II
13 microspheres were dissolved in 1 ml DMF then analysed using gas
14 chromatography and comparing peak heights to external standards of either
15 acetone or heptane diluted in DMF with 10 mgm of blank microspheres.
16 The results are expressed as percent by weight.

17 Microbial load. One hundred mgm of CFA/II microsphere(single
18 dose) are suspended in 2 ml of sterile saline than poured into 2 blood agar
19 plates (1 ml each). All colonies are counted and identified after 48 hours in
20 culture at 37° C and expressed as total number. Similiar amount of
21 microspheres is in 0.25 ml aliquots poured onto 4 different fungal culture
22 plates (Sabharagar, casein peptone agar with chloramphenicol, brain heart
23 infusion agar with chloramphenol and genimycin or chloramphenicol alone)

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1 and cultured at 30° for 5 weeks and the colonies counted & identified and
2 expressed as total number.

3 CFA/II Release From Microsphere Study. Thirty mgm samples in
4 triplicate were placed in 2 ml conical upright microcentrifuge tubes containing
5 1 ml of PBS at pH 7.4. The tubes were capped and kept immerized in a water
6 bath maintained at 37° C with constant agitation. The samples were withdrawn
7 at 1, 3, 6, 8, 15 and 22 hour time intervals by centrifuging the sample tubes
8 for 5 minutes at the maximum speed of bench top centrifuge. The release
9 medium was collected through a 5 um nylon screen for CFA/II protein analysis
10 using the micro BCA method and comparing results to BSA standard and
11 expressing results as percent cumulative release of CFA/II.

12 General Safety Test. Two doses of one hundred mgm CFA/II
13 microspheres were suspended by sonication for 5 minutes in 3.1 mls of sterile
14 vaccine diluent consisting of injectable saline containing 0.5% Polysorbate 60[®]
15 N.F., 0.03 ml were injected intraperitoneally into each of 2 mice and 3 mls
16 were administered by gastric lavage to each of 2 guinea pigs. The animals
17 were weighed both before and at 7 days following the vaccine administration.
18 All animals were observed daily for any signs of toxicity.

19 Rabbits. 1.5-2 kilogram male specific pathogen free New Zealand
20 white rabbits, obtained from closed colony maintained at the National Institute
21 of Health, Bethesda, MD. They were selected for study if they did not have
22 measurable serum antibodies at 1:2 dilution to CFA/II antigens by ELISA and
23 were not colonized by E. coli as determined by culture of rectal swabs.

Intra-Muscular Immunization of Rabbits and ELISA. Two Rabbits

were immunized with CFA/II microsphere vaccine at 25 ug protein in two different sites intra-muscularly on day 0. Sera were obtained from all animals before immunization on day 0 and days 7 and 14. The sera were tested by ELISA for IgG antibodies to CFA/II antigen and individual coli surface (CS) proteins CS3 and CS1. ELISA plates were coated with 3 ug/ml of either CFA/II antigen, CS3 or CS1 protein (150 ul/well) and incubated with 150 ul/well of PBS with 0.1% BSA for four hours at room temperature. The PBS with 0.1% BSA is washed out with PBS and 100 ul/well of different dilutions of each rabbit serum in triplicate was added to the plates. The dilutions ranged from undiluted to 1:1,000,00. The plates were incubated with the sera for 3 hours at 37° C. The sera were washed out with PBS and then horse radish peroxidase-conjugated goat anti- rabbit IgG was added to the plates at a 1:1000 dilution (100 ul/well). The plates were incubated for 1 hour at room temperature with the peroxidase conjugate. The conjugates were washed out of the plates with PBS and 100 ul/well of an ABTS substrate solution (Kikegaard and Perry Laboratories) was added to each well in the plates. The plates were read using the ELISA reader(Dynatech Laboratories MR 580) at a wave length of 405 nm after 15 minutes.

The results are measured and expressed as antibody titers.

Intra-duodenal Vaccination of Rabbits. Rabbits (N=5) were

vaccinated with CFA/II microspheres containing either 25 or 50 ug of protein suspended in 1 ml of PBS containing 0.5% Polysorbate 60 on day 0 and 7 by sonication. The microspheres were injected through an Olympus BF type P10

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1 bronchoscope into the duodenum of the rabbits following sedation with an intra
2 muscular injection of ketamine HCl (50 mgm I.M.)(Ketaset, Fort Dodge
3 Laboratories, Fort Dodge, IA) and Lylazine (10 mgm I.M.) (Rompom Malay
4 Corporation, Shnanee, KS). The endoscope was advanced ready under direct
5 vision into the stomach which was insufflated with a 50 ml bolus of room air
6 via a catheter passed through the biopsy channel. The catheter was advanced
7 through the pylorus 3-4 cm into the duodenum and the microsphere suspension
8 in 1 ml of PBS was injected, followed by a 9 ml flush of PBS and removal of
9 the air bolus. The rabbits were sacrificed by chemical euthanasia at day 14.

10 Anti-CFA/II Stimulated Lymphocytoid Transformation. The Peyer's
11 Patches were removed and cell suspension obtained by teasing and irrigation
12 with a 20 gauge needle and syringe. The cells were placed in 2 ml of media at
13 a concentration of 2.5×10^6 cells/ml for each well of a 24 well plate. These
14 cells were challenged separately with BSA and the CFA/II antigen at doses of
15 500, 50 and 5 ng/ml in triplicate wells. The plates were incubated at 37° C
16 with 5% CO₂. On day 4 the cells were mixed while still inside the wells and
17 100 ul were transferred into each of 4 wells in a 96 well flat bottom
18 microculture plate. Thus, the challenge at each antigen dose represented by 3
19 wells in the 24 well plate is now represented by 12 wells in the 96 well plate.
20 After the cells have been transferred, each well is pulsed with 20ul of 50
21 uCi/ml tritiated thymidine. These pulsed plates were incubated for 6 hrs then
22 harvested with 96 Mach II Cell harvested (Tourtec, Inc.). The lymphocyte
23 proliferation was determined by the tritiated thymidine incorporation measured
24 in kilo counts per minute (Kcpm) using the 1205 Beta Plate Liquid scintillation

counter (LKB, Wallac, Inc.). The results are expressed as mean Kcpm \pm SD and compared to media controls.

Anti-CFA/II Antibody Secreting B Cells. Spleen cells were obtained

from immunized rabbits on day 14 following intra-duodenal immunization with CFA/II microsphere vaccine. The cells were placed in 96 well round bottom microculture plate at a final concentration of 6×10^5 cells/well and incubated for 0, 1, 2, 3, 4 and 5 days at 37° C with 5 CO₂. 96 well flat bottom microculture plates were coated with 3 ug/ml of CFA/II antigen overnight blocked with PBS with 0.05% Polysorbate 60°. On the harvest days, the cells were gently flushed out of the wells of the round bottom plates and transferred to the corresponding well in the antigen coated, 96 well flat bottom microculture plates to be tested for the presence of antibody secreting cells using ELISPOT technique. The plates were incubated with the cells overnight at 4° C. The cells were then washed out of the flat bottom plates with PBS, and 100 ul/well of horserudish-peroxidase conjugated, goat anti-rabbit total antibody (IgM, IgG, and IgA) at a 1:1000 dilution were added to the plates. The Plates were incubated for 1 hour at room temperature, at which time, the conjugate was washed out of the plates with PBS. 0.1 mgm of agarose was dissolved in 10 ml of PBS by boiling. After the agar solution cooled but not hardened, 6 mgm of 4-chloro-naphthol, 2 mls of methanol and 30 ul of hydrogen peroxide were added to make the substrate solution. The solution was placed into the flat bottom plates (100 ul/well) and the plates were held at 4° C overnight so the agar could harden. The number of browish spots per 15

1 wells (total of 9×10^6 spleen cells) was counted and represents the number of
2 antibody secreting cells per 9×10^6 spleen cells.

3 Pathological Evaluation. Rabbits were euthanized by parenteral
4 overdose of sodium pentobarbital and were subjected to complete
5 necropsy. Sample of tissue including small and large intestine
6 with gut associated lymphoid tissue, spleen, mesenteric and mediastinal lymph
7 nodes, lung, trachea, liver and kidney were fixed by immersion in 10% neutral
8 buffered formalin. Tissues were routinely processed for light microscopy and
9 embedded in paraffin. Five micron thick sections were stained with
10 hematoxylin and eosin.

11 Statistical Analysis. The paired student t-test was used to determine p
12 values.

13 RESULTS

14 Particle Size Distribution. The results of size frequency analysis of
15 150 randomly chosen microspheres are shown in (Figure 37). The particle
16 size distribution is plotted in % frequency against particle size in diameter
17 (size) expressed in μm . The average number frequency diameter is 4.6 μm .
18 The average volume frequency diameter is 4.6 μm . The percent volume
19 between diameters of 5-10 μm is 63% and the percent volume less than 10 μm
20 diameter is 88%.

21 Scanning Electron Microscopy. The microspheres are seen in
22 (Figure 38) which is a scanning electron photomicrograph. Nearly all the

1 microspheres are less than 10 um as compared to the 5 um bar. Also the
2 surfaces of the microsphere are smooth and demonstrate lack of pores.

3 Protein Content. The protein loads of the individual batches are the
4 following: K62A8, 1.16% \pm 0.10 SD; K63A8, 1.023% \pm 0.17SD; K64A8,
5 1.232% \pm 0.13 SD; and K65A8, 0.966% \pm 0.128 SD. The mean
6 average protein load is 1.16% \pm 0.15 SD. The protein load of the CFA/II
7 microsphere vaccine in the final dose vial is the following: Lot L74F2,
8 1.175% \pm 0.17SD.

9 Moisture Content. The CFA/II microsphere vaccine (Lot 74F2)
10 percent water content was found using the Karl Fischer titrimeter method to be
11 2.154% using triplicate samples.

12 Acetonitrile and Heptane Residuals. The acetonitrile residuals of the 4
13 individual CFA/II microsphere batches are the following: K62A8, <0.1%;
14 K62A8, <0.1%, K64A8, <0.1%; and K65A8, <0.1%. The acetonitrile
15 residual of the CFA/II microsphere vaccine in the final dose vial is the
16 following: Lot L74F2, 0.07 \pm 0.05%. The heptane residual of the 4
17 individual CFA/II microsphere batches are the following: K62A8, 1.9%;
18 K63A8, 1.4%; K64A8, 1.6% and K65A8, 1.6%. Following pooling in
19 heptane and subsequent drying, the heptane residual of the CFA/II microsphere
20 vaccine in the final dose vial is the following: Lot L74F2, 1.6 \pm 0.1%.

21 Microbial load. One hundred milligrams (a single dose) of CFA/II
22 microsphere vaccine (Lot L74F2) in the final dose vial was suspended in a 2
23 ml of sterile saline and 1 ml poured onto a blood agar culture plate x 2.
24 Twenty two colonies grew after 48 hours of culture and 21 were identified as

1 coagulase negative staphylococcus and 1 as a micrococcus species. All these
2 bacteria are considered to be nonpathogenic to humans. An additional 100
3 mgms of CFA/II microsphere vaccine (Lot L74F2) were suspended in 2 ml of
4 sterile saline and 0.25 ml poured onto four different fungal culture agars
5 and cultered for 5 weeks. Three fungal colonies grew and each was identified
6 as A. glaucus.

7 CFA Release From Microsphere Study. Three thirty mgm samples
8 were incubated each in 1 ml of PBS, pH 7.4 at 37° C for 0, 1, 3, 6, 8, 15 and
9 22 hours. The superanates were removed and replaced at these times. The
10 protein content was determined for each supernate sample and the results are
11 seen in (Figure # 39). The results are plotted as percent release of CFA/II
12 against time in hours. An average of 8% of CFA/II is released at one hour
13 rising to 20% at 8 hours then a slower release to 25% at 22 hours.

14 General Safety Test. Two one hundred milligrams(a single dose) of
15 CFA/II microsphere vaccine in the final dose vials were suspended in 3.1 mls
16 of the sterile dilulents consisting of 0.85 N saline prepared for injection plus
17 Polysorbate 60^a at 0.5%. Two Swiss mice (16.5 gm) were injected
18 intraperitoneally with 0.03 mls and two Hartley guinea pigs (350 gm) were
19 administered by gastric lavage 3.0 mls.

20 None of these animals displayed any signs of toxicity for 7 days. The
21 mice gained and average of 2.3 gms and the guinea pigs gained and average,
22 of 43 grams. The CFA/II microsphere vaccine therefore passed the general
23 safety test.

1 Serum IgG Antibody Responses. Tw rabbits were immunized in two
2 separate sites intra-muscularly with 25 ug of protein of CFA/II microsphere
3 vaccine (Lot L74F2) in the final dose vial. Sera samples were obtained before
4 and 7 and 14 days following immunization. The IgG antibody titers to CFA/II
5 CSI and CS3 protein were determined using ELISA and the results seen in
6 (Figure 32). The results are expressed as mean antibody titers against the
7 different antigens at 0, 7 and 14 days. High antibody titers greater than 1000
8 were seen at 7 days to both CS1 and CS3 protein which rose to greater than
9 10,000 by day 14. The individuals titers to CFA/II are seen in (Figure 33).
10 Rabbit 109 developed an antibody titer of 1,000 by day 7 rising to 3,000 by
11 day 14. Rabbit 108 had a log higher rise at day 7 and 2 log higher rise at day
12 14 being 3×10^4 at day 7 going to 1×10^5 at day 14.

13 Anti-CFA/II Stimulated Lymphocyte Transformation. Five rabbits
14 were immunized intra-duodenally with CFA/II microspheres containing either
15 25 ug of protein (human dose equivalent) or 50 ug of protein on days 0 and 7
16 and then sacrificed on day 14. The Peyer's patch lymphocytes were
17 challenged in vitro with CFA/II antigen, BSA media and alone. The
18 lymphocyte transformation was determined by tritiated thymidine
19 incorporation. The results of the high dose immunization are seen in (Figure
20 34). The results are expressed as Kcpm against antigen dose. No response to
21 BSA or media control is seen in any of the five rabbits. All rabbits responded
22 by lymphocyte transformation in a dose dependent manner to the CFA/II.

23 The highest dose responses were 3-10X's the media control are highly
24 significant with a p value of <0.002 . The results of the 5 rabbits receiving

1 the low dose immunization are seen in (Figures 35). Rabbit #80 gave no
2 response probably due to poor Peyer's patch cell population which did not
3 respond were to Concanavallin A mitogenic stimulation either. The remaining
4 4 rabbits gave positive responses with the high CFA/II dose response being 2-
5 8x media control and highly significant with p values of <0.009 . Again no
6 response were seen to BSA compared to the media cont

7 Anti-CFA/II Antibody Secreting B-Cells Five rabbits immunized
8 intraduodenally with CFA/II microsphere containing 50 ug of CFA/II protein
9 at days 0, 7 than sacrificed at day 14 were studied. The spleen cells were
10 placed into microculture then ELISPOT forming B-Cells secreting specific anti
11 CFA/II antibody determined at days 0, 1, 2, 3, 4 and 5. The results are seen
12 in (Figure 36) and expressed as # of antibody secreting cells per 9×10^6 spleen
13 cell against culture days. Positive responses were seen in all 5 rabbits on days
14 2-5. Days of maximum responses occurred on day 3 for rabbits 65 and 66;
15 day 4 for rabbit 85; and day 5 for rabbits 83 and 86. The responses are
16 highly significant being 7-115 times higher than the 1-2 cells seen on all days
17 in 4 control rabbit (67, 69, 72, 89) (Figure 45). Here is a composite graph
18 expressing the mean counts \pm ISD for all days of culture.

19 Pathological Evaluation. A consistent finding in the spleens of all
20 rabbits both the 25 and 50 ug protein dose groups was minimal to mild diffuse
21 lymphocytic hyperplasia the periarteriolar lymphatic sheaths (T cell dependent
22 areas). Two of five rabbits of the 50 ug dose group (#83 and #86) also had
23 mild lymphocytic hyperplasia of splenic follicular (B cell dependent) areas.

1 The three rabbits in an untreated control group had histologically normal
2 spleens.

3 Reactive hyperplasia of mesenteric lymph nodes was often seen in
4 vaccinated rabbits. Two of five rabbits in the 25 ug dose equivalent group
5 (#83 and #86) also had minimal to mild lymphocytic hyperplasia of cortical
6 follicular (B cell dependent) areas. The mesenteric lymph nodes of the other
7 vaccinated rabbits and of the untreated control rabbits were within normal
8 limits. Incidental or background lesions found in one or more rabbits of all
9 three group were acute minimal to mild pneumonia and foreign body
10 microgranulomas of the cecal gut associated lymphoid tissue.

11 Discussion

12 McQueen et al (33) has found that the AF/R1 adhesin of rabbit diarrheagenic
13 Escherichia coli (RDEC-1) incorporated into biodegradable microspheres could
14 function as a safe and effective oral intestinal vaccine in the rabbit diarrhea
15 model. The AF/R1 was incorporated into poly D,L-lactide-co-glycolide)
16 microspheres and administered intraduodenally. Jarboe et al (34) reported that

17 Peyer's patch cells obtained from rabbits immunized intra-duodenally
18 with AF/R1 in microspheres responded with lymphocyte proliferation upon in
19 vitro challenge with AF/R1. This early response at 14 days gave a clear
20 indication as to the immunogenicity of E. coli pili contained within the
21 polymer microspheres.

22 In developing an effective oral vaccine against enterotoxigenic E. coli,
23 CFA/II pili given as an oral vaccine was found to be ineffective. The CFA/II
24 pilus proteins were found to be rapidly degraded when treated with 0.1M HCl

1 and pepsin conditions mimicking those contained in the stomach (27). The
2 CFA/II was found to be immunogenic when given in high doses intra-
3 intestinally producing intestinal secretory IgA antibodies (26).

4 The CFA/II vaccine has now been incorporated into poly(D,L lactide-
5 co-glycolide) microspheres under Good Manufacturing Practices and tested
6 under Good Laboratory Practices. The microspheres, are spherical, smooth
7 surfaced and without pores. The majority (63%) are between 5-10 um in
8 diameter by volume. This size range has been suggested to promote
9 localization within the Peyer's patch in mice and perhaps enhance local
10 immunization (29-32). The protein content being 1.174% is close to 1%
11 which was the goal of the vaccine formulation. One percent was chosen
12 because 0.62% was the core loading of the AF/R1 microspheres which were
13 effective. Also a small percentage perhaps 1-5% (35) is anticipated to be
14 taken up from the intestine, a higher protein content would lead to considerable
15 loss of protein.

16 The organic residuals are of course a concern. Heptane exposure
17 would be 1.7 mgm per vaccine dose. This is compared to the occupational
18 maximum allowable exposure of 1800 mgm/15 min. Therefore, the heptane
19 contained with the CFA/II microsphere vaccine appears to be a safe level.
20 The acetonitrile is very low - 0.1 mgm per vaccine dose. The human oral
21 TDLO is 570 mgm\Kg (any non lethal toxicity). Therefore, the acetonitrile
22 contained with the CFA/II microsphere vaccine appears to be at a safe level.
23 The CFA/II vaccine was produced under sterile conditions. However, the
24 process of incorporation of the desalted CFA/II vaccine into the polymer

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(HBsAg) after a single injection of vaccine. In use studies, the 50% effective dose (ED_{50}) for the alum precipitated Heptavax B vaccine (Merck, Sharp and Dohme) was 3.8 ng when administered in a 3 injection regimen, but was 130 ng when one immunizing dose was used. Antigen release studies revealed that HBsAg is bound tightly to the alum, indicating that the antigen remains in situ until scavenged by phagocytic cells. the ED_{50} with a 3 dose regimen of aqueous HBsAg was 180 ng, as opposed to over 2000 ng for daily injections of low doses for 90 days and 240 ng for a regimen that employed initially high doses that decreased geometrically at 3 day intervals over 90 days. The ED_{50} was 220 ng for a single dose regimen of HBsAg microencapsulated in poly (DL-lactide-co-glycolide) in a form that was too large to be phagocytized and had an antigen release profile similar to that achieved with the geometrically decreasing regimen of doses. This indicates that single injection of microencapsulated immunogens can achieve similar effects in vivo to those achieved with multiple dose regimens. For HBsAg the effect to be achieved appears to be 3 pulses of particulate immunogens that can be scavenged by phagocytes.

INTRODUCTION

A major disadvantage of inactivated vaccines lies in their inability to confer lasting immunity. Due to rapid elimination from the body, multiple doses and boosters are usually required for continued protection³⁷. Alum adjuvants, achieving their effects by mechanisms of antigen presentation and

1 sustained antigen release³⁸, have been used successfully to increase the potency
2 of several inactivated vaccines including those against tetanus, anthrax, and
3 serum hepatitis^{39,40}. Though useful, alum preparations are deficient in several
4 aspects. Control over quantity and rate of antigen release is limited, often
5 resulting in a continued requirement for immunization schedules consisting of
6 multiple injections given over a period of several months to years. Alum
7 adjuvants are also non-biodegradable and thus remain within the body, serving
8 as a nidus for scar tissue formation³⁸ long after they have served their function.

9 Protracted, multiple immunization schedules are unacceptable during
10 massive mobilization and deployment of troops. Changing global disease
11 patterns and deployment of new biological warfare agents by enemy forces
12 require flexibility in the number and types of vaccine antigen administered to
13 soldiers departing for combat. Any immunization schedule requiring
14 completion during engagement in non-linear combat would compromise this
15 flexibility and place an unreasonable burden on our health care delivery
16 system.

17 The main objective of this study was, therefore, to develop a
18 biodegradable, controlled-release adjuvant system capable of eliminating the
19 need for multistep vaccination schedules. This investigation was designed to :
20 (1) determine in an animal model hepatitis B vaccine release rate
21 characteristics desirable for single-step immunization, (2) incorporate those
22 release rate characteristics into a one-step biodegradable poly(DL-lactide-co-
23 glycolide)(DL-PLG) microencapsulated hepatitis B surface antigen (HBsAg)
24 vaccine, and (3) conduct an in vivo trial comparing the effectiveness of this

- 1 single-step vaccine against the conventional three-step hepatitis vaccine
- 2 currently employed⁴¹. The results were intended to provide the foundation for
- 3 further development of single step vaccines against hepatitis and other
- 4 militarily significant diseases⁴².

MATERIALS AND METHODS

Vaccine potency assay. Due to its availability, compatibility with cage mates, and potential application to the study of hepatitis B vaccine⁴³, the female Walter Reed (ICR) strain mouse was used. A hepatitis B vaccine potency assay for comparing the six-month immunization schedule currently in use⁴¹ with that of a single-step immunization by sustained antigen release was established according to the following protocol: Specimens for baseline antibody titers were collected from twenty mice by exsanguination. Immediately prior to exsanguination, all mice employed in this and other exsanguination procedures in these studies were anesthetized with a 0.1 ml injection of V-Pento. Groups of 12 mice were then immunized according to a schedule consisting of either 0.25 ug, 0.025 ug, 2.5 ng, 0.25 ng, 2.5 pg, or 0.25 pg Heptavax B vaccine (HBV) administered in 50 microliter volumes subcutaneously (s.c.) at the beginning and end of the first, and end of the second month of the protocol. Antibody responses to the vaccine were monitored immediately before the third injection and approximately one month after the third injection. Specimens for antibody determination were collected by exsanguination of seven anesthetized mice from each group and assayed along with the baseline samples by the Abbott Ausab radioimmunoassay. Percent seroconversion verses micrograms vaccine employed with calculated by the method of Reed and Muench⁴³. These data were employed to establish a mouse vaccine potency assay calibrated to detect differences between Heptavax B and other forms of hepatitis b vaccine.

1 In vitro antigen release rate from Heptavax B vaccine. Antigen
2 release from aluminum hydroxide adjuvant in HBV was measured by pumping
3 2 cc per hour of 1:20,000 thimerosal in saline at 4°C across a 0.2 µ pore
4 diameter Acrodisc filter apparatus containing 20 µg of vaccine. The effluent,
5 collected by a Gilford fraction collector, was assayed periodically over several
6 weeks for protein by UV absorption at 280 nm on a Beckman model 25 double
7 beam spectrophotometer, and for HBsAg by the Abbot Ausria II
8 radioimmunoassay made quantitative by using HBsAg standards supplied by
9 Merk, Sharp, and Dohme. Accuracy of the HBsAg standards were verified by
10 Biuret protein determination and by UV absorbance at 215 nm and 225 nm⁴⁴.
11 Nonspecific antigen retention on the Acrodisc filter was assessed by measuring
12 percent recovery of a known quantity of HBsAg. Spontaneous degradation of
13 vaccine antigen was monitored by comparing daily ratios of antigen to total
14 protein detected in the effluent.

15 Evaluation of HBsAg stability. These studies were designed to
16 characterize the stability of the aqueous antigen to the various physical
17 conditions employed in the microencapsulation process. Conditions tested
18 included lyophilization with reconstitution in distilled water, cyclohexane,
19 methylene chloride, chloroform, methyl alcohol, acetone, iso-octane, hexane,
20 acetone, pentane, or heptane; irradiation while lyophilized; and, exposure to
21 elevated temperatures. Samples exposed to organic solvents were first
22 lyophilized, reconstituted with the test solvent, evaporated to dryness under
23 nitrogen at room temperature and reconstituted with distilled water. Test
24 samples were compared against untreated controls by assaying serial dilutions

1 of each with the Abbot Ausria II procedure and comparing the plots of counts
2 per minute versus dilution.

3 Assessment of the effect of antigen release rate on vaccine potency.

4 Three regimens simulating patterns of free HBsAg release that could be
5 achieved by microencapsulation were contrasted with the three monthly dose
6 regimen of Heptavax B for immunizing mice. To do so, 24 ICR mice were
7 divided into groups and vaccinated as indicated below. Seven mice from each
8 subgroup were exsanguinated at the end of the second and third months of the
9 experiment. The sera were separated and assayed for specific antibody
10 response to HBsAg by Abbot Ausab procedure.

11 HV regimen a: 14 mice/treatment receiving 3 s.c. injections of 250,
12 25, 2.5 or 0.25 ng doses of HBV a month apart.

13 HBsAg regimen a: 14 mice/treatment receiving 3 s.c. injections of
14 250, 25, 2.5 or 0.25 ng doses of aqueous HBsAg a month apart.

15 HBsAg regimen b: 14 mice/treatment receiving total doses of 750, 75,
16 7.5 or 0.75 ng of aqueous HBsAg over 3 months by s.c. injections of ZX_y ng
17 at 3 day intervals, where Z is the total dose, y is the injection number, and X
18 is the fraction indicated on the graph in Fig. 1 minus the fraction for the
19 previous injection.

20 HBsAg regimen c: 14 mice/treatment receiving daily s.c. injections of
21 8.33, 0.833, 0.0833 or 0.00833 ng of aqueous HBsAg for 3 months.

22 Microencapsulation in DL-PLG. Microencapsulated immunogens
23 were fabricated by Southern Research Institute, Birmingham, AL. DL-PLG
24 polymers were synthesized from the cyclic diesters, DL lactide and glycolide,

1 by using a ring-opening melt polymerization catalyzed by tetraphenyl tin⁴⁵.
2 The resulting polymer was dissolved i methylene chloride, filtered free of
3 insoluble contaminants and precipitated in methanol. Lactide-co-glycolide
4 mole ration of the product was determined by nuclear magnetic resonance
5 spectroscopy. Encapsulation of HBsAg in DL:PLG polymer was achieved by
6 an organic phase separation process⁴⁶. Microcapsules of the desired size
7 (approximately 100 micron diameter in these studies) were isolated from each
8 batch by wet sieving with hexane through standard mesh stainless steel sieves
9 and then dried for 24 hours in a vacuum chamber maintained at room
10 temperature.

11 In vitro analysis of encapsulated antigens. Integrity of encapsulated
12 antigen was assessed by comparing the antigen to total protein ratios present in
13 microcapsule hydrolysates with those obtained from suspensions of pure
14 unencapsulated antigen. Centrifuge tubes containing 1 ug of either
15 microencapsulated or pure vaccine antigen in 1 ml saline were incubated at 4°C
16 with shaking. Samples were collected at weekly intervals by interrupting the
17 incubation, sedimenting the contents of the tubes by centrifugation and
18 withdrawing the supernates. Sediments were resuspended in 200 microliters of
19 saline and supernates were assayed for HBsAg by the Abbott Ausria II
20 radioimmunoassay. The HBsAg standard described earlier in this report was
21 used as the calibrator. Antigen destruction due to the encapsulation procedure
22 was monitored by a comparison between the antigen assayed from the
23 hydrolysate and from the untreated antigen control.

1 Assessment of the potency of DL:PLG microencapsulated HBsAg for
2 immunizing ICR mice when used alone and in combination with Heptavax B
3 vaccine. HBsAg loaded microcapsules that had been fabricated by Southern
4 Research Institute to release the majority of their HBsAg load within 40 to 50
5 days were serially diluted in 10-fold steps by mixing the dry, loaded capsules
6 with blank placebo capsules of similar size and composition. The resulting
7 stock and diluted microcapsule preparations were placed onto lyophilizer when
8 not in use in order to assure minimum spontaneous degradation prior to
9 injection. On the day of injection, a predetermined weight of microcapsules or
10 placebo-diluted microcapsules was added to each syringe. Immediately prior
11 to injection either one or two ml of injection vehicle (2 wt % carboxymethyl
12 cellulose and 1 wt % Tween 240 in water, Southern Research Institute) were
13 drawn into the microcapsule-loaded syringes, mixed and injected. All mice
14 were vaccinated s.c. as indicated below:

15 Group 1: 14 mice/treatment receiving 25, 25, 2.5, 0.25 or 0.925 ng
16 HBV.

17 Group 2: 14 mice/treatment receiving 1000, 250, 25 or 2.5 ng
18 aqueous HBsAg with Bovine Serum Albumin (BSA).

19 Group 3: 7 mice receiving 1600 ng microencapsulated HBsAg
20 (HBsAg) plus 0.25 ng HBV and 14 mice/treatment receiving 160, 16, 1.6 or
21 0.16 ng HBsAg plus 0.25 ng HBV.

22 Group 4: 7 mice receiving 1600 ng HBsAg plus 2.5 ng HBV and 14
23 mice/treatment receiving 160, 16, 1.6 or 0.16 ng HBsAg plus 2.5 ng HBV.

Group 6: 7 mice receiving 2500 ng HBsAg and 14 mice-treatment receiving 250, 25, 2.5 or 0.25 ng HBsAg. Fifty-three days after receiving the above injections, the mice were anesthetized with an 0.1 cc injection of V-Pento and exsanguinated. Blood samples were allowed clot and the sera were separated by centrifugation. The serum samples were assayed for antibody to HBsAg by the Abbott Ausab procedure.

RESULTS

Heptavax B vaccine potency. As can be seen from Table 4, the total dose of vaccine which produced seroconversion in 50% of

TABLE 1.2 Potency of Heptavax B vaccine in ICR mice.

No.	ng Heptavax B per Injection								ED ₅₀
Inj.	250	25	2.5	.25	.025	.0025	.00025	ng	
2	5/5	4/4	3/6	2/6	0/5	1/4	0/4		1.7
3	6/6	6/6	4/6	1/6	0/6	1/6	1/6		2.0

* Number positive seroconversions per number vaccinated.

The vaccinated mice (ED_{50}) for HBV was approximately 2 ng, whether the vaccine was given in 2 or 3 injections.

1 In vitro antigen release rate from HBV. HBsAg release from the 20
2 ug of Heptavax was not detected in any of the 21 fractions of saline collected
3 from the Acrodisc polycarbonate filter over a 30 day period. The lower limit
4 of detection for the Abbott Auria II assay employed was approximately 4.8
5 ng/ml. The Acrodisc filter used in the antigen release study was back-washed
6 with 10 mls normal saline. Quantitation of the HBsAg present within this
7 back-wash eluent revealed the presence of the original 40 ug of Heptavax
8 vaccine which had been loaded into the filter at the start of the experiment.
9 This is the concentration which one would expect to obtain if there had been
10 no deterioration of the original 40 ug/ml HBsAg loaded onto the filter, none of
11 the antigen eluted from the alum adjuvant, and none of the vaccine had
12 adsorbed onto or passed through the filter.

13 Evaluation of antigen stability. Considerable effort was expended in
14 assessing the effects of physical conditions on the antigenicity of HBsAg to
15 insure that the conditions used for microencapsulation would not cause serious
16 degradation of the immunogen. Since microencapsulation must be performed
17 on dried materials which are suspended in organic solvents, the HBsAg, which
18 was provided as a solution, had to be lyophilized. Initial attempts at
19 lyophilizing HBsAg in normal saline resulted in a total loss of detectable
20 antigen within samples. Dilution of the HBsAg sample 1:10 in distilled water
21 prior to freezing resulted in reservation of nearly 100% of the antigen
22 detectable in the original sample. Studies of antigen stability at elevated
23 temperature revealed that HBsAg may be heated to 50°C for up to one hour
24 without appreciable loss of antigen. The studies involving exposure of

1 lyophilized antigen to organic solvents indicated that iso-octane and hexane had
2 minimal effects on antigenicity, but that 95% to 100% of antigenicity was lost
3 upon exposure to either methylene chloride, chloroform, cyclohexane, or
4 methyl alcohol. Moderate antigen loss occurred in the presence of acetone,
5 pentane and heptane. As a result of these studies, hexane was chosen as the
6 solvent for microencapsulation.

7 Assessment of the effect of antigen release rate on vaccine potency.

8 The results (Table 3) indicated that immunogen formation (i.e., the alum
9 adjuvant of Heptavax B) had far more

TABLE 13 Effect of immunogen formulation and vaccination regimen on potency for immunizing ICR mice.

Immunogen Formulation	Regiment	ng Total Dose HBsAg				ED ₅₀
		750	75	7.5	.75	ng
Heptavax B	a	7/7*	6/6	5/7	1/7	3.8
Aqu. HBsAg	a	4/6	3/7	0/7	0/6	180
Aqu. HBsAg	b	6/7	0/7	1/7	0/7	240
Aqu. HBsAg	c	1/7	0/7	0/7	0/7	>2000

* Number positive seroconversions per number vaccinated.

a 3 injections of 1/3 total dose a month apart.

b Injections administered every three days for 90 days in decreasing dosages according to a logarithmic progression.

c Injections of 1/90 total dose daily for 90 days.

effect on potency than did the vaccination regimen, and that pulsing with large doses of immunogen was more effective than continuous administration of small doses.

HBsAg release from DL:PLG microcapsules. The microcapsules employed in this study were designed to disintegrate within three weeks after hydration. It is evident from the release curve (Fig. 10) that they performed as designed, releasing approximately 17% of their total load in an initial pulse

and approximately 7% of the remaining available HBsAg over the first three weeks.

Assessment of the potency of DL:PLG microencapsulated HBsAg for immunizing ICR mice when used alone and in combination with Heptavax B vaccine. The results (Table 14) indicate that the microencapsulated HBsAg had approximately the same immunogenicity as did the Heptavax B. Neither immunogens were sufficiently potent to effect with a singly injection seroconversion rates similar to those achieved after three injections of Heptavax B (Table 12). Only the immunogen

TABLE 14 Potencies of Heptavax B and microencapsulated HBsAg by single injection S.C. when administered alone and in combination to immunize ICR mice.

Var. Dose	ng Const.	ng Variable Dose			Var. Dose Tot. Dose	
Immunogen Dose mHBsAg	2500	250	25	2.5	.25	ED ₅₀ ng ED ₅₀ ng
Heptavax B	0	13/14*	8/14	4/14	0/13	130 130
Heptavax B	0.16		11/13	4/14	1/14	1.7 1.8
Heptavax B	1.6		10/13	1/14	0/13	100 100
Heptavax B	16		3/14	1/14	1/14	>470 >490
Heptavax B	160		3/12	2/11	1/12	>370 >530
Heptavax B	1600		7/7	7/7	7/7	<0.8 1600
Mic. HBsAg	0	3/6	6/15	1/13	2/10	2/14 220 220

* Number positive seroconversions per number vaccinated.

1 combination of Heptavax B with 0.16 ng mHGSAg provided this level of
2 seroconversion. At the ED_{50} endpoint, the 0.16 ng dose of mHGSAg is
3 approximately 10% of the total dose. Similarly, a small amount of Heptavax
4 B appeared to enhance the immunogenicity of the microencapsulated
5 immunogen, although the combination was clearly less immunogenic when the
6 two formulations were present at equivalent concentrations.

7 DISCUSSION

8 The potential advantage of microcapsules lies in their ability to be
9 programmed during fabrication into forms that have quite different release
10 profiles, including slow and steady release, multiple bursts of antigen over a
11 period of time, or combinations of release forms. Sieving allows choice of
12 microcapsule size, and the ability of DL-PLG to sequester antigen from the
13 host's immune system until release occurs enhances control over exposure of
14 the recipient's immune system to antigen over a sustained period of time.
15 These characteristics provided the impetus for these studies as they indicate
16 potential for achieving the effects of a multiple injection regimen by
17 controlling release in vivo after a single injection.

18 The results of these studies are important for gaining an under
19 standing of the fundamental differences between the manner in which alum and
20 microcapsules interact with the immune system. The antigen release studies
21 showed that alum firmly bound the antigen on its surface, whereas the
22 microcapsules sequestered the antigen load within the interstices of an
23 immunologically inert polymer. Release of antigen from microcapsules was
24 spontaneous and gradual while antigen release from alum was probably

1 enzymatically mediated within host macrophages. Alum thus performed at
2 least two useful functions as an adjuvant: by bearing its entire load of antigen
3 upon its surface, it provided a large single exposure of antigen to the host;
4 and, by being readily phagocytized by host macrophages, it served as a means
5 of targeting the antigen to the immune system.

6 In order for microcapsules to be efficacious as a vaccine delivery
7 system, a means of incorporating the two properties common to alum adjuvant
8 must be devised. These properties, which were discussed above, are targeting
9 antigen to the immune system and delivering the antigen load in a single
10 concentrated pulse at its target. A gradual, sustained release of free antigen,
11 as was achieved with the 100 micron microcapsules used in these studies,
12 could be expected to elicit an immune response similar to that seen with either
13 regimen b or regimen c (Table 13), where multiple injections of small doses
14 were employed. In fact, as shown in Table 11, the microencapsulated
15 immunogen elicited a response similar to that achieved with regimen b. This
16 is probably due to the fact that the microcapsules release approximately 10%
17 of their antigenic load immediately after injection.

18 Microcapsules with extended release patterns tend to be large (> 10
19 microns in diameter) and thus fail to be readily phagocytized. In order for the
20 larger microcapsules with prolonged antigen release characteristics to be
21 efficacious, the antigen eventually released from those microcapsules would
22 have to be in a form which targeted and concentrated it within the recipient's
23 immune system. This might be effectively achieved by microencapsulation of

1 antigen coated alum or by microencapsulating clusters of smaller (<10
2 microns) microcapsules.

3 Microcapsules under 10 microns in diameter tend to be readily
4 phagocytized and also tend to undergo rapid spontaneous degradation due to
5 their high surface to volume ratio. These smaller microcapsules would be well
6 suited for eliciting a primary response if their pulse of antigen release could be
7 programmed to occur after phagocytosis.

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PHASE III

This phase of the invention relates to providing novel biocompatible and biodegradable microspheres for burst-free programmable sustained release of biologically active agents, inclusive of polypeptides, over a period of up to 100 days in an aqueous physiological environment. Potentially release period is capable of being further modulated beyond 100 days to about 365 days by careful selection of a blend of uncapped and end-capped biodegradable-biocompatible copolymer and molecular weights.

Several publications and patents are available for sustained
5 release of active agents from biodegradable polymers,
particularly, poly(lactide/glycolides) (PLGA). Prior usages of
PLGA for controlled release of polypeptides have involved the use
of molar ratios of lactide/glycolide (L/G) of 75/25 to 100/0 for
molecular weights <20,000. Further prior art preparations of PLGA
utilized fillers or additives in the inner aqueous layer to
improve the stability and encapsulation efficiency and/or to
increase the viscosity of the aqueous layer, thereby modulating
polymer hydrolysis and the biologically active agent or
polypeptide release.

In addition, the prior art use of PLGA copolymers were end-
capped, in that the terminal carboxyl end groups were blocked. In
these end-capped co-polymers, the microcapsule preparations
exhibited a low to moderate burst release of ~ 10-40% of the
20 entrapped polypeptide in the first 24 hours after placement in an
aqueous physiological environment. In part, these characteristics
are due to the use of fillers in the inner aqueous phase.
Further, a 1-month release of polypeptide is known with the use
of a 75/25 co-polymer of PLGA of Mw <20,000.

25 Investigations in controlled release research has been
proceeding especially to obtain a 1 to 2 month delivery system

for biologically active agents or polypeptides using poly(lactide/glycolide) polymers. However, most of these systems have one or more of the following problems: Poor encapsulation efficiency and large 'burst release' followed by an intermediate 'no release' or 'lag phase' until the polymer degrades. In general, release from these polymers occur over a period from about 4 weeks to about several months. In addition, in order to achieve this release a 50/50 copolymer of MW > 30,000 or a 75/25 copolymer of MW > 10,000 are employed which often results in residual polymer remaining at the site of administration long after the release of active core.

This invention provides biocompatible and biodegradable microspheres that have been designed for novel, burst free, programmable sustained release of biologically active agents, including polypeptides over a period of up to 100 days in an aqueous physiological environment.

Unlike currently available release systems, which rely on the use of fillers/additives such as gelatin, albumin, dextran, pectin, polyvinyl pyrrolidone, polyethylene glycol, sugars, etc., and are still prone to low encapsulation efficiencies and "burst effects", this invention achieves high encapsulation and "burst-free" release without the use of any additive. In this invention, burst-free, programmable sustained release is achieved through the use of a unique blend of the 'uncapped' and end-capped forms of poly(lactide/glycolide) polymer in the molecular weight range of 2,000 to 60,000 daltons.

In general, microspheres described in this invention are produced by a unique emulsification technique wherein an inner water-in-oil (w/o) emulsion is stabilized by dispersing in a solvent-saturated aqueous phase containing an emulsion stabilizer. A ternary w/o/w emulsion is then formed by emulsifying the above w/o emulsions in an external pre-cooled aqueous phase containing an o/w emulsifier. Essentially, the

- 2 -

inner w/o emulsion is comprised of an aqueous layer containing from ~ 2 to about 20% (w/w) of the active agent to be entrapped and an oil layer containing poly(lactide/glycolide) copolymer in concentrations ranging from ~ 5 to about-- 50% (w/w oil phase).

5 The copolymer includes molecular weight ranging from 2,000 to about 60,000 daltons, with molar composition of lactide/glycolide from 90/10 to 40/60 and a blend of its uncapped and end-capped forms in a ratio of 100/0 to 1/99. Very high encapsulation efficiencies of about 80 to 100% are achieved depending on polymer molecular weight and structural form.

Programmable release of active core over variable durations between 1-100 days is achieved by a judicious selection of process parameters such as polymer concentration, peptide concentration and the aqueous/oil phase ratio.

This invention is particularly suitable for high encapsulation efficiencies and burst-free, continuous programmable release of polypeptides of molecular weights ranging from 1,000 to about 250,000 daltons, and also other biologically active agents over a period of 1-100 days. A uniqueness of the invention is that when using a 100/0 blend of the uncapped and capped polymer, the final phase of active core release is concurrent with the complete solubilization of the polymer to innocuous components, such as lactic and glycolic acids. This is a significant advantage over the currently available 30 day -
25 release systems wherein a major regulatory concern is about toxicity of residual polymer at the site of administration, long

20 This invention relates to the design of biocompatible and biodegradable microspheres for novel, programmable sustained release of biologically active agents, including polypeptides over a period of up to 100 days in an aqueous physiological environment with little or no burst release.

Unlike currently available release systems which rely on the use of fillers/additives such as gelatin, albumin, dextran, pectin, polyvinyl pyrrolidone, polyethylene glycol, sugars, etc., and are still prone to low encapsulation efficiencies and "burst effects", this invention achieves high encapsulation efficiency after release of the active core.

5 The microcapsules described in this invention are suitable for administration via several routes such as parenteral (intramuscular, subcutaneous), oral, topical, nasal, rectal and vaginal routes.

and 'burst-free' release without the use of any additive. In this invention, burst-free, programmable sustained release is achieved through the use of a unique blend of the 'uncapped' and end-capped forms of poly (lactide/glycolide) polymer.

5 The 'uncapped' form refers to "poly(lactide/glycolide) with free carboxyl end groups" which renders the polymer more hydrophilic compared to the routinely used end-capped form. Currently used 'end-capped' polymer hydrates between 4-12 weeks depending on the molecular weight, resulting in an intermediate 'no release' or a 'lag phase'. The uncapped polymer hydrates typically between 5 to 60 days depending on the molecular weight, thus releasing its core continuously without a lag phase. A careful blend of the two forms and appropriate molecular weights and L/G ratios, results in a continuous release between 1 to 100 days. In addition, release within this time is programmable by a judicious selection of process parameters such as polymer concentration, peptide concentration and the aqueous/oil phase ratio.

20 The copolymer in this invention includes molecular weight ranging from 2,000 to 60,000 daltons, a lactide/glycolide ratio of 90/10 to 40/60 and a blend of the uncapped/capped forms in the ratio of 100/0 to 1/99. The molecular weight of the polypeptide may be in the range of 1000 to 250,000 daltons while that of other biologically active agents may range from 100 to 100,000
25 daltons.

Microcapsules described in this invention are prepared by a

unique aqueous emulsification technique which has been developed for use with the uncapped polymer to provide superior sphere morphology, sphere integrity and narrow size distribution. This is accomplished by first preparing an inner water-in-oil (w/o) by
5 mixing the solutions of polymer in an organic solvent, such as methylene chloride and the biologically active agent in water. This is followed by stabilization of the w/o emulsion in a solvent-saturated aqueous solution containing an o/w emulsifier such as polyvinyl alcohol. A ternary emulsion is then formed by emulsifying the w/o emulsion in an external aqueous phase containing the same emulsifier as above at concentrations ranging from 0.25 - 1% w/v. Microcapsules are hardened upon solvent removal by evaporation, rinsed to remove residual emulsifier and lyophilized. Low temperature is used both at the time of primary emulsification (w/o emulsion formation) and during the formation of the final w/o/w emulsion to achieve stable emulsion and superior sphere characteristics.

In the context of the invention, a biologically active agent is any water-soluble hormone drugs, antibiotics, antitumor
20 agents, antiinflammatory agents, antipyretics, analgesics, antitussives, expectorants, sedatives, muscle relaxants, antiepileptics, antiulcer agents, antidepressants, antiallergic drugs, cardiotonics, antiarrhythmic drugs, vasodilators, antihypertensives, diuretics, anticoagulants, antinarcotics,
25 and the agents listed in the summary of the invention section herein

More precisely, applicants have discovered a pharmaceutical composition and process with the following itemized features:

1. A controlled release microcapsule pharmaceutical formulation, which
5 may contain a pharmaceutically-acceptable adjuvant, for burst-free, sustained, programmable release of a biologically active agent over a duration from 1-100 days, comprising an active agent and a blend of uncapped and end-capped biodegradable poly(lactide/glycolide).

2. The pharmaceutical formulation of item 1, wherein the biodegradable poly(lactide/glycolide) is a blend of uncapped and capped forms, in ratios ranging from 100/0 to 1/99.

3. The microcapsules of items 1 or 2 wherein the copolymer (lactide to glycolide L/G) ratio for uncapped and endcapped polymer is 52/48 to 48/52.

4. The microcapsules of items 1 or 2 wherein the copolymer L/G ratio for uncapped and end-capped polymer is 90/10 to 40/60.

5. The microcapsules of items 1 or 2 or 3 or 4 wherein the molecular weight of the copolymer is between 2,000-60,000 daltons.

20 6. The microcapsules of items 1 or 2 or 3 or 4 or 5 wherein the biologically active agent is a peptide or polypeptide.

7. The microcapsules of item 6, wherein said polypeptide is histatin consisting of 12 amino acids and having a molecular weight of 1563.

25 8. The microcapsules of items 1 or 2 or 3 or 4 or 5 or 6 characterized by the capacity to completely release histatin in

an aqueous physiological environment from 1-35 days with a 100/0 blend of uncapped and end-capped poly(lactide/glycolide) having a L/G ratio of 48/52 to 52/48, and a molecular weight <15,000.

5 9. The microcapsules of items 1 or 2 or 3 or 4 or 5 or 6 characterized by the capacity to completely release histatin in an aqueous physiological environment from 18-40 days with a 100/0 blend of uncapped and end-capped poly(lactide/glycolide) having a L/G ratio of 48/52 to 52/48 and a molecular weight range of 28,000-40,000.

10. The microcapsules of items 1 or 2 or 3 or 4 or 5 or 6 characterized by the capacity to release up to 90% of the histatin in an aqueous physiological environment from 28-70 days with a 0/100 blend of uncapped and end-capped poly(lactide/glycolide) having a L/G ratio of 48/52 to 52/48 and a molecular weight range of 10,000-40,000 daltons.

11. The microcapsules of items 1 or 2 or 3 or 4 or 5 or 6 characterized by the capacity to release up to 80% of histatin in an aqueous physiological environment from 56-100 days with a 0/100 blend of uncapped and end-capped poly(lactide/glycolide) 20 having a L/G ratio of 75/25 and a molecular weight of < 15,000 daltons.

12. The microcapsules of items 7 or 8 or 9 or 10 or 11 having analogs of histatin with chain lengths of from 11-24 amino acids of molecular weights from 1,500-3,000 daltons and 25 characterized by the following structures:

1. D S H A K R H H G Y K R K F H E K H H S H R G Y

2. K R H H G Y K R K F H E K H H S H R G Y R
3. K R H H G Y K R K F H E K H H S H R
4. R K F H E K H H S H R G Y R
5. A K R H H G Y K R K F H
6. *A K R H H G Y K R K F H
7. K R H H G Y K R K F

* D-amino acid

13. The microcapsules of items 1 or 2 or 3 or 4 or 5 wherein the biologically active agent is a polypeptide Leutinizing hormone releasing hormone (LHRH) that is a decapeptide of molecular weight 1182 in its acetate form, and having the structure:

p- E H W S Y G L R P G

14. The microcapsule of items 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 having a molecular weight of from 1,000 to 250,000 daltons.

15. The microcapsules of items 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 wherein release profiles of variable rates and durations are achieved by blending uncapped and capped microspheres as a cocktail in variable amounts.

16. The microcapsules of items 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 wherein release of profiles of variable rates and duration are achieved by blending uncapped and capped polymer in different ratios within the same microshrerers.

17. The microcapsules of items 6 or 7 or 8 or 9 or 10 or 11

or 12 or 13 or 14 or 15 or 16 wherein the entrapped polypeptide is any of the vaccine agents against enterotoxigenic E. coli (ETEC) such as CFA/I, CFA/II, CS1, CS3, CS6 and CS17 and other ETEC-related enterotoxins.

5 18. The microcapsules of items 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 wherein the entrapped polypeptide consists of peptide antigens of molecular weight range of about 800-5000 daltons for immunization against enterotoxigenic E. coli (ETEC).

19. The microcapsules of items 1 or 2 or 3 or 4 or 5 wherein said biologically active agents are selected from the group consisting of water-soluble hormone drugs, antibiotics, antitumor agents, anti inflammatory agents, antipyretics, analgesics, antitussives, expectorants, sedatives, muscle relaxants, antiepileptics, antiulcer agents, antidepressants, antiallergic drugs, cardiotonics, antiarrhythmic drugs, vasodilators, antihypertensives, diuretics, anticoagulants, and antinarcotics, in the molecular weight range of 100-100,000 daltons.

20 20. The microcapsules of items 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 wherein said biodegradable poly(lactide/glycolide) is in an oil phase, and is present in about 1-50% (w/w).

25 21. The microcapsules of items 1 or 2 or 3 or 4 or 5 or 6 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 wherein concentration of the active agent is in the range of 0.1 to about 60% (w/w).

22. The microcapsules of items 1 or 2 or 3 or 4 or 5 or 6 or

8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 wherein a ratio of the inner aqueous to oil phases is about 1/4 to 1/40(v/v).

23. A process for preparing controlled release microcapsule formulations characterized by burst-free, sustained, programmable release of biologically active agents comprising: Dissolving biodegradable poly (lactide/glycolide), in uncapped form in methylene chloride, and dissolving a biologically active agent or active core in water; adding the aqueous layer to the polymer solution and emulsifying to provide an inner water-in-oil (w/o) emulsion; stabilizing the w/o emulsion in a solvent-saturated aqueous phase containing a oil-in-water (o/w) emulsifier; adding said w/o emulsion to an external aqueous layer containing oil-in-water emulsifier to form a ternary emulsion; and stirring the resulting water-in-oil-in-water (w/o/w) emulsion for sufficient time to remove said solvent, and rinsing hardened microcapsules with water and lyophilizing said hardened microcapsules.

24. A process for preparing controlled release microcapsule formulations characterized by burst-free, sustained, programmable release of biologically active agents comprising:

dissolving biodegradable poly(lactide/glycolide) in end-capped form in methylene chloride, and dissolving a biologically active agent or active core in water; adding the aqueous layer to the polymer solution and emulsifying to provide an inner water-in-oil emulsion; stabilizing the w/o emulsion in a solvent-saturated aqueous phase containing a oil-in-water (o/w)

emulsifier; adding said w/o emulsion to an external aqueous layer containing oil-in-water emulsifier to form a ternary emulsion; and stirring a resulting water-in-oil-water (w/o/w) emulsion for sufficient time to remove said solvent; and rinsing hardened
5 microcapsules with water; and lyophilizing said hardened microcapsules.

25. The process of items 23 or 24 wherein a solvent-saturated external aqueous phase is added to emulsify the inner w/o emulsion prior to addition of the external aqueous layer, to provide microcapsules of narrow size distribution range between 0.05-500 μ m.

26. The process of items 23 or 24, wherein a low temperature of about 0-4°C is provided during preparation of the inner w/o emulsion, and a low temperature of about 4-20°C is provided during preparation of the w/o/w emulsion to provide a stable emulsion and high encapsulation efficiency.

27. The process of items wherein a 100/0 blend of uncapped and end-capped polymer is used to provide release of the active core in a continuous and sustained manner without a lag phase.

20 28. The microcapsules of items 6, wherein, when the entrapped polypeptide is active at a low pH, such as LHRH, adrenocorticotrophic hormone, epidermal growth factor, calcitonin released polypeptide is bioactive.

25 29. The microcapsules of items 6 or 7 or 8 or 9 or 10 or 11, wherein, when entrapped peptide such as histatin is inactive at a low pH, a pH-stabilizing agent of inorganic salts are added to

the inner aqueous phase to maintain biological activity of the released peptide.

30. The microcapsules of items 6 or 7 or 8 or 9 or 10 or 11 wherein, when entrapped polypeptide such as histatin is inactive at a low pH, a non-ionic surfactant such as polyoxyethylene sorbitan fatty acid esters (Tween 80, Tween 60 and Tween 20) and polyoxyethylene - polyoxypropylene block copolymers (Pluronic) is added to the inner aqueous phase to maintain biological activity of the released polypeptide.

31. The microcapsules of items 29, wherein placebo spheres loaded with the pH-stabilizing agents are coadministered with polypeptide-loaded spheres to maintain the solution pH around the microcapsules and preserve the biological activity of the released peptide in instances where the addition of pH-stabilizing agents in the inner aqueous phase is undesirable for the successful encapsulation of the acid pH sensitive polypeptide.

32. The microcapsules of item 30 wherein placebo spheres loaded with non-ionic surfactant are coadministered with polypeptide-loaded spheres to maintain biological activity of the released peptide where the addition of non-ionic surfactants in the inner aqueous phase is undesirable for successful encapsulation of the acid pH sensitive polypeptide.

33. The microcapsules of items 1 or 2 or 3 or 4 or 5 or 6 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 comprising a blend of uncapped and capped polymer, wherein complete

solubilization or the copolymer leaves no residual polymer at the site of administration and occurs concurrently with the complete release of the entrapped agent.

5 34. A process of using microcapsules of items 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 for human administration via parenteral routes, such as intramuscular and subcutaneous.

35. A process of using microcapsules of items 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 for human administration via topical route.

36. A process of using microcapsules of items 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 for human administration via oral routes.

37. A process of using microcapsules of items 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 for human administration via nasal, transdermal, rectal, and vaginal routes.

Conservation of bioactivity of polypeptides

As the polymer degrades rapidly, there is a precipitous drop in pH accompanied by the release of soluble oligomers in the microenvironment which may affect the biological activity of acid pH-sensitive peptides/proteins. In such instances, biological activity can be maintained by the use of inorganic salts or buffering agents in the inner aqueous phase codissolved with the peptide.

The following unique advantages are characteristics of this invention:

1. Burst-free, prolonged, sustained release of polypeptides and other biologically-active agents from biocompatible and biodegradable microcapsules up to 100 days in an aqueous physiological environment without the use of additives in the core.

2. Release of active core programmable for variable durations over 1-100 days, by using a blend of uncapped and capped polymer of different molecular weights and copolymer ratio, and by manipulating the process parameters.

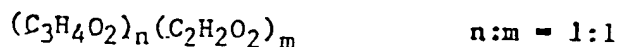
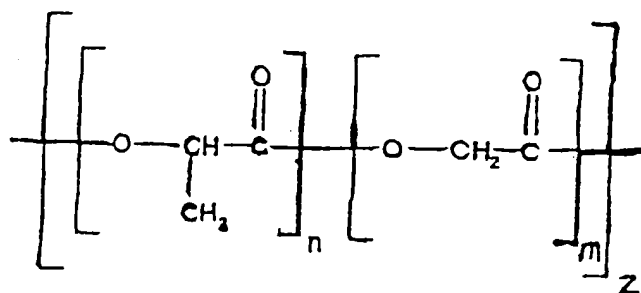
3. Complete release of the active core is concurrent with complete solubilization of the carrier polymer to innocuous components, such as lactic and glycolic acids, especially when using a 100/0 blend of uncapped/capped polymer. This is of tremendous significance, as most biodegradable polymers currently used for 1-30 day delivery, do not degrade completely at the end of the intended release duration, thereby causing serious concern

or regulatory authorities on the effects of residual polymer at the site of administration.

4. Ease of administration of the microcapsules in various dosage forms via several routes, such as parenteral
- 5 (intramuscular and subcutaneous), oral, topical, nasal, vaginal, etc.

The hydrophilic homo-and co-polymers based on D,L-lactide and glycolide contains hydrophilic adjusted homo-and co-polymers with free carboxylic end groups, and is characterized by the formula:

Poly(D,L-lactide-co-glycolide) 50:50



Wherein Z= Molecular Weight/130; for example Z=92 for Mw 12,000 and 262 for Mw 34,000.

20. While the molar ratio of the lactide to glycolide may vary, it is most preferred that the lactide to glycolide copolymer ratio be 50:50.

25. Reference is now made to FIG.48 which depicts a blood-drug concentration versus time graph that shows conventional drug administration using a series of dosages compared to an ideal controlled release system. Unfortunately, many drugs have a

blending of the two forms in a single formulation comprising different ratios of uncapped to capped polymer, would significantly influence the polymer hydration and hence release of the active core thereby providing release curves of any desirable pattern. Manipulation of polymer hydration and degradation resulting in modulation of release of active core is achieved by the addition of uncapped polymer to end-capped polymer in amounts as low as 1% up to 100%.

While referring to Table 14 in conjunction with FIG. 50, it can be seen that the cumulative Histatin release from PLGA microspheres from several batches prepared using 50/50 and 75/25 uncapped and end-capped, polymer modulates release between 1 to 100 days by varying the process parameters. 1-35 days by uncapped 50/50, 18-56 days by capped 50/50 and 56-100 days by capped 75/25.

In referring to FIG. 51, a view is provided through a scanning electron micrograph of PLGA microspheres designed for a one to two month release system prepared using end-capped polymer of Mw 30-40k daltons.

FIG. 52 depicts the cumulative Histatin release from PLGA microspheres, in which the release profiles are from several batches prepared using 50/50, uncapped and capped polymer, and varying the process parameters to modulate release between 28 to 60 days.

Figure 53 represents cumulative Histatin release from PLGA microspheres --- these combined release profiles are from several batches prepared using 50/50 uncapped and capped polymer, and varying the process parameters to modulate release between 1-60 days.

In the context of the invention, a biologically active agent is any water-soluble antibiotics, antitumor agents, antipyretics analgesics, anti-inflammatory agents, antitussives, expectorants, sedatives, muscle relaxants, anti epileptics, antiulcer agents,

anti-depressants, anti-allergic drugs, cardiotonics, antiarrhythmics drugs, vasodilators, antihypertensives, diuretics, anticoagulants, hormone drugs, anti-narcotics, etc.

5 In general, "burst free" sustained release delivery of biologically active agents from PLGA microspheres is accomplished in the context of this invention using of 90/10 to 40/60 molar ratios, and ratios of uncapped polymer to end-capped polymer of 100/0 to 1/99.

10 In general, the approaches for designing the biologically active agents encapsulated in the uncapped and combination uncapped/end-capped PLGA microspheres and characteristics of these encapsulants are briefly set forth below as follows:

1. Providing PLGA microspheres of surface morphologies using 50/50 uncapped and capped polymers of Mw ~ 8-40K daltons as shown in Figs. 49 and 51.

2. Providing in vitro release of a polypeptide, Histatin from PLGA microspheres, as shown in Figs. 50 and 52, using uncapped and capped polymer of Mw ~ 8-40K daltons and molar ratios such as 50/50 and 75/25.

For example, design of a 1-12 week bioactive compound release system is achieved using PLGA with the following specifications:

- 30
1. Polymer molecular weight:
 - about 2-60K daltons
 2. Copolymer molar ratio (L/G):
 - 90/10 to 40/60
 3. Polymer end groups:
 - uncapped and /or end-capped

and combining judiciously within the following parameters:

- 35
4. Polymer concentration
 - from 5 to 50%
 5. Inner aqueous to oil phase ratio:
 - 1:5 to 1:20 (v/v)
 6. Peptide loads:
 - from 2 to about 40% (w/w polymer)
- 40

and by using the unique aqueous emulsification method described in the invention.

The uniqueness and novelty of invention may generally be summarized in a brief way as follows:

1. Use of uncapped poly(lactide/glycolide) to achieve burst-free, continuous, sustained, programmable release of biologically active agents over 1-100 days.

2. Use of a unique aqueous emulsification system to achieve superior microsphere characteristics such as uniform sphere morphology and narrow size distribution.

3. Burst-free, prolonged, sustained release of polypeptides and other biologically active agents from biocompatible and biodegradable microcapsules up to 100 days in an aqueous physiological environment without the use of additives in the inner core.

4. Release of active core programmable for variable durations over 1-100 days by using a blend of uncapped and capped polymer for different molecular weights and copolymer ratios and manipulating the process parameters.

5. Complete release of the active core concurrent with complete solubilization of carrier polymer to innocuous components such as lactic and glycolic acids, especially when using a 100/0 blend of uncapped/capped polymer. This is of tremendous significance as most biodegradable polymers currently in use for 1-30 day delivery, do not degrade completely at the end of the intended release duration causing serious concern for regulatory authorities on the effects of residual polymer at the site of administration.

6. Ease of administration of the microcapsules in various dosage forms via several routes such as parenteral (intramuscular and subcutaneous), oral, topical, nasal, vaginal, etc.

The following examples are illustrative of, but not limitations upon the microcapsule compositions pertaining to this invention.

Example 12

Poly(lactide/glycolide) (PLGA) microcapsules are prepared by a unique aqueous emulsification technique which has been developed for use with the uncapped polymer to provide superior sphere morphology, sphere integrity and narrow size distribution (See Figures 32 and 32a). This is accomplished by dissolving the polymer in a chlorinated hydrocarbon solvent such as methylene chloride and dissolving the biologically active agent in water. A

w/o emulsion is then formed by mixing the solutions of polymer and the active agent by sonication, followed by emulsion stabilization in a solvent - saturated aqueous solution containing polyvinyl alcohol. A ternary emulsion is then formed by emulsifying the w/o emulsion in an external, pre-cooled aqueous phase containing polyvinyl alcohol (0.25 - 1% w/v). Microcapsules are hardened upon removal of solvent by evaporation, rinsed to remove any residual emulsifier, and then lyophilized.

Table 14 lists the microcapsule compositions, Nos. 1-21 thus prepared, consisting of a biologically active polypeptide, Histatin (composed of 12 amino acids and a molecular weight of 1563) and blends of uncapped and capped polymer of ratios 100/0 to 1/99, and having a lactide/glycolide ratio of 90/10 to 40/60, and a molecular weight range between 2000 to 60,000 daltons.

Example 13

Microcapsule compositions are prepared as described in Example 1 wherein the copolymer L/G ratio is 48/52 to 52/48, and the ratio of uncapped/capped polymer is 100/0. The active core is Histatin (Mw 1563), the polymer molecular weight is < 15,000 and the polymer concentrations vary from 7% to ~ 40% w/w. Compositions 1, 2, 4, 12-14 and 16-18 are listed in Table 14.

Release profiles of the active core from the compositions in an aqueous physiological environment, such as phosphate-buffered saline, pH 7.0 maintained at $37 \pm 1^\circ\text{C}$ are plotted as cumulative percentage release versus time, and presented in Figure 50.

Burst-free, variable release from 1-35 days is achieved by varying the polymer concentration from 7 to ~ 40% w/w in the oil phase.

Example 14

Microcapsule compositions are prepared as described in Example 2, wherein the aqueous /oil ratio is varied from 1/4 to 1/20 (v/v). Compositions 1, 2, 4 and 12 are listed in Table 14.

Release profiles of the active core from the compositions in an aqueous physiological environment described in Example 2 are plotted as cumulative percentage release versus time, and presented in Figure 50.

Burst-free, continuous release from 1-35 days, with different onset and completion times are achieved by selecting

different w/o ratios in the inner core.

Example 15

Microcapsule compositions are prepared as described in Example 2, wherein the polymer molecular weight is 28,000-40,000 and polymer concentrations vary from 5% to ~ 15% w/w. Compositions 19-21 are listed in Table 14.

Release profiles of the active core from the compositions in an aqueous physiological environment are described in Example 2 are plotted as cumulative percentage release versus time and presented in Figure 52.

Burst-free, variable release from 18-40 days is achieved by varying the polymer concentration.

Example 16

Microcapsule compositions are prepared as described in Example 2, wherein the ratio of uncapped/capped polymer is 1/99 and polymer concentrations vary between 5% to ~ 12% w/w. Compositions 10 and 11 are listed in Table 14.

Release profiles of the active core from the compositions in an aqueous physiological environment are described in Example 2, and plotted as cumulative percentage release versus time and presented in Figure 50.

Burst-free, variable release from 28-70 days is achieved by varying the polymer concentration in the oil phase.

Example 17

Microcapsule compositions are prepared as described in Example 5, wherein polymer molecular weight is 28,000-40,000 and polymer concentrations vary between 5% to ~ 12% w/w. Compositions 5 and 6 are listed in Table 14.

Release profiles of the active core from the compositions in an aqueous physiological environment are described in Example 2 and are plotted as cumulative percentage release versus time, and presented in Figure 52.

Burst-free, variable release from 28-70 days is achieved by varying the polymer concentration.

Example 18

Microcapsule compositions are prepared as described in Example 6, wherein the aqueous/oil ratio varies between 1/5 to

1/25 (v/v). Compositions 3 and 7 are listed in Table 14.

Release profiles of the active core from the compositions in an aqueous physiological environment are described in Example 2, and plotted as cumulative percentage release versus time, and presented in Figure 52

Burst-free, variable release from 28-70 days is achieved by varying the aqueous/oil ratios.

Example 19

Microcapsule compositions are prepared as described in Example 5, wherein the copolymer ratio is 75/25 and polymer concentrations vary between 5% to ~ 25% w/w. Compositions 8 and 9 are listed in Table 1.

Release profiles of the active core from the compositions in an aqueous physiological environment are described in Example 2, and are plotted as cumulative percentage release versus time, and presented in Figure 50.

Burst-free, variable release from 56->90 days is achieved by varying the polymer concentration in the oil phase.

Example 20

Microcapsule compositions are described in Example 2, wherein the active core is leutinizing hormone releasing hormone (LHRH, a decapeptide of molecular weight 1182) and the polymer concentration is ~40% w/w. Release profiles of the active core from the composition in an aqueous physiological environment is described in Example 2, and is plotted as cumulative percentage release versus time, and presented in Figure 54

Burst-free, continuous and complete release is achieved within 35 days, similar to Histatin acetate.

Example 21

Microcapsule compositions are prepared as described in Example 2, wherein an additive such as sodium salt (carbonate or bicarbonate) is added to the inner aqueous phase at concentrations of 1-10% w/w to maintain the biological activity of the released polypeptide.

Burst-free, variable release from 1-28 days is achieved similar to Examples 2 & 3, and the released polypeptide is biologically active until 30 days, due to the presence of the sodium salt.

Example 22

Microcapsule compositions are prepared as described in Example 2, wherein an additive such as a nonionic surfactant, polyoxyethylene/polyoxypropylene block copolymer (Pluronic F68 and F127) is added to either the inner oil or the aqueous phase at concentrations from 10-100% w/w, to maintain the biological activity of the released polypeptide.

Burst-free, continuous release from 1-35 days is achieved similar to Examples 2 & 3, and the released polypeptide is bioactive due to the presence of the surfactant.

Example 23

Cumulative histatin release from the microcapsule compositions described in Examples 1 through 11 and release profiles plotted in Figures 49 and 50 show the burst-free, programmable peptide release for variable duration from 1-100 days. Virtually any pattern of cumulative release is achievable over a 100 day duration by a judicious blending of several compositions, as shown in Figure 53.